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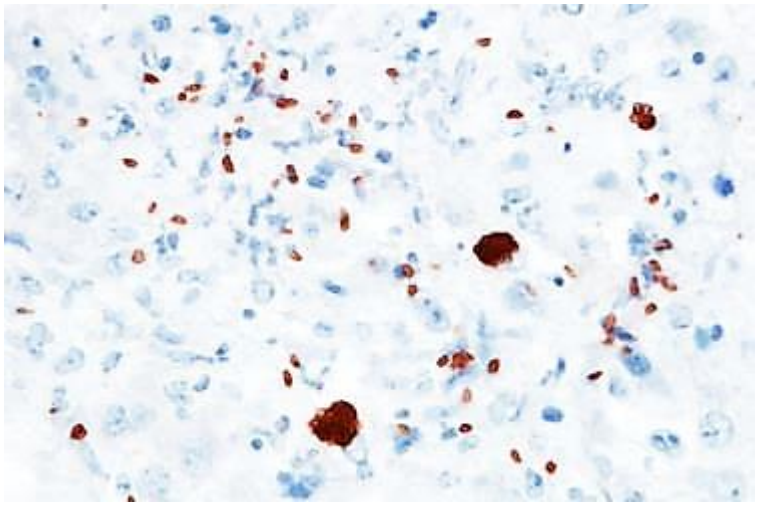
Specific diagnostic tools for protozoan infection of ruminants



By **Tanja Lepore**

Student number: s1473490

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zoetis

Lay summary

Neospora caninum, *Toxoplasma gondii* and *Sarcocystis* spp. are closely related protozoan parasites known to cause abortions in livestock raising major concerns for animal welfare, production inefficiency and economic losses. Although abortion is a major problem for livestock operations and animal welfare worldwide, the identification of a specific cause is particularly difficult and achieved in less than 50% of the cases, even in well-established diagnostic laboratories.

Neospora, *Toxoplasma*, and *Sarcocystis* spp. share many common morphological and biological similarities making differentiation through immunological and molecular methods between these protozoan parasites challenging. Aetiological diagnostics tests are required to confirm the presence of association of disease with the specific parasites, and to determine the cause of abortion to adopt the most relevant disease control strategy. As such, it is necessary to have access to specific diagnostic tools to confirm or rule out the presence of *T. gondii, N. caninum,* and *Sarcocystis* spp. as the cause of abortion in mammalian species. Previous diagnostics using immunohistochemical (IHC) analysis with polyclonal sera have shown unspecific as cross-reactivity between these parasites occurred and *Neospora*, *Toxoplasma*, and *Sarcocystis* spp. could not be easily differentiated and detected. Moreover, the differentiation and identification of one or multiple parasite species from fixed tissue samples has shown to be difficult using molecular methods.

The primary aim of this PhD was to develop a genus-specific PCR assay for the detection and identification of *N. caninum, T. gondii* and *Sarcocystis* spp. using fixed and fresh tissue material. PCR primers were developed from target regions (18S rRNA gene and ITS1 region) and the PCR assay was standardized (tested for specificity and sensitivity) and validated. The PCR assay allowed detection and identification of protozoan parasites. The second aim was to develop genus-specific antibodies (polyclonal and monoclonal) raised against recombinant proteins of *N. caninum*, *T. gondii* and *Sarcocystis* spp. to enable specific detection and identification. This was completed by the development of target recombinant proteins for *Neospora* (rNcSRS2 and rNcSAG1) and for *Toxoplasma* (rTgSRS2) using bacterial expression system. These recombinant proteins were used to develop polyclonal three polyclonal sera

(anti-*Neospora* NcSRS2, anti-*Neospora* NcSAG1 and anti-*Toxoplasma* TgSRS2). The IHC assay using each polyclonal sera was standardized and validated. Each polyclonal sera was shown to be specific, and results showed that the sera can be used in immunohistochemical detection of the parasite on formalin fixed paraffin embedded samples. Lastly, a *Neospora* monoclonal antibody ME7.1.B12.C9 was successful produced that was able to specifically detect *Neospora* in fixed tissue sections. This study shows the development and improvement of diagnostic analysis using genus-specific PCR assays and genus-specific antibodies to be used for the identification and detection of *Neospora*, *Toxoplasma* and *Sarcocystis* in formalin fixed paraffin embedded samples.

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Authors declaration

I hereby declare that this thesis has been composed solely of my own work and all results presented therein have been conducted by the author between November 2014 and October 2018, under the supervision of Dr. Francesca Chianini, Dr. Frank Katzer (Moredun Research Institute) and Dr. Alastair Macrae (University of Edinburgh). This work has not been submitted, in whole or in part, in any previous application for a degree or professional qualification. Except where stated otherwise by reference or acknowledgement, the work presented is entirely my own.

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Tanja Lepore

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Abstract

Neospora caninum, *Toxoplasma gondii* and *Sarcocystis* spp. are closely related intracellular protozoan parasites causing neosporosis, toxoplasmosis and sarcocystosis, respectively. *Toxoplasma* and *Neospora* are major causes of abortion in livestock worldwide leading to substantial economic losses. *Toxoplasma* is a well-known infectious parasite of sheep and a wide range of warm-blooded animals, including humans. *Neospora* predominantly causes disease in cattle, although infections in other warm-blooded animals have been found to cause disease. *Sarcocystis* infect a wide range of intermediate and definitive mammalian hosts. Although abortion is a major problem for livestock operations and animal welfare worldwide, the identification of a specific cause is particularly difficult and achieved in less than 50% of the cases, even in well-established diagnostic laboratories.

Neospora, *Toxoplasma*, and *Sarcocystis* spp. share many common morphological and biological similarities making differentiation through immunological methods between these protozoan parasites challenging. Aetiological diagnostics tests are required to confirm the presence of association of disease with specific parasites, and to determine the cause of abortion to adopt the most relevant disease control strategy. As such, it is necessary to have access to specific diagnostic tools to confirm or rule out the presence of *Toxoplasma gondii*, *Neospora caninum*, and *Sarcocystis* spp. as the cause of abortion in mammalian species.

The primary aim of this PhD was to develop a genus-specific PCR assay for the detection of *N. caninum*, *T. gondii* and *Sarcocystis* spp. using fixed and fresh tissue material. Suitable target regions for the production of genus-specific PCR primers were identified using the 18S rRNA gene and ITS1 regions. Primers were tested for sensitivity and specificity using DNA from various protozoan parasites. For the 18S rRNA gene, general PCR primers were developed to amplify DNA from *Neospora*, *Toxoplasma* and *Sarcocystis* spp. 18S group-specific primers were developed to enable detection of *T. gondii* and *N. caninum* from *Sarcocystis* spp. Several 18S *Sarcocystis* specific group primers were developed to enable differentiation of a variety of *Sarcocystis* spp. Species-specific primers were developed using the ITS1 region to enable diagnosis of *Neospora* from *Toxoplasma*. PCR assays were standardized, and

primers were validated using DNA extracted from fixed and fresh tissues to enable the diagnosis of different protozoan species.

The second aim was to develop genus-specific antibodies (polyclonal and monoclonal) raised against recombinant proteins of *N. caninum, T. gondii* and *Sarcocystis* spp. to enable specific diagnosis. For this aim, suitable target genes for the production of recombinant proteins for *Neospora, Toxoplasma* and *Sarcocystis* spp. were identified. Recombinant proteins were expressed using *E. coli* and analysed for cross-reactivity. Three recombinant proteins for *Neospora*, three for *Toxoplasma* and one for *Sarcocystis* were successfully expressed. Of those, two recombinant proteins for *Neospora* (rNcSRS2 and rNcSAG1) and one recombinant protein for *Toxoplasma* (rTgSRS2) were used for subsequent antibody production.

For the development of polyclonal antisera, rabbit pre-immune sera were tested to choose the best candidate for immunisation using the recombinant proteins. Polyclonal sera were tested using immunohistochemistry for functionality and specificity. The polyclonal antibodies were validated using a range of ruminant clinical cases suspected of protozoal infections. Based on the recombinant protein expression the best candidates were taken forward for the development of a genus-specific monoclonal antibody. For this study, three rabbits were chosen for immunisation using recombinant proteins, and three polyclonal rabbit sera (anti- *Neospora* NcSAG1, anti-*Neospora*-NcSRS2, anti- *Toxoplasma* TgSRS2) were generated. Each polyclonal sera was shown to be specific, and results showed that the sera can be used in immunohistochemical detection of the parasite on formalin fixed paraffin embedded samples.

For monoclonal antibody production, mice were immunised with recombinant proteins NcSRS2 and TgSRS2. Hybridoma clones were generated, and clones that showed reactivity and specificity using ELISA and immunohistochemistry were selected to produce monoclonal antibodies. The study achieved the successful production of *Neospora* monoclonal antibody ME7.1.B12.C9. No *Toxoplasma* specific monoclonal antibody was produced. This study shows that the genus-specific PCR assays and genus-specific antibodies can be used for the identification of *Neospora, Toxoplasma* and *Sarcocystis* in formalin fixed paraffin embedded samples.

Chapter 1. General Introduction

1.1. The Apicomplexa parasites *T. gondii, N. caninum* and *Sarcocystis* spp.

Apicomplexa are intracellular protozoan parasites, containing up to 2.1 million species, of which the most eminent Sarcocystidae are *Toxoplasma gondii*, *Neospora caninum* and various *Sarcocystis* spp. (Adl et al., 2007). *Toxoplasma*, *Neospora* and *Sarcocystis* are known to cause toxoplasmosis, neosporosis and sarcocystosis, respectively. *Toxoplasma* and *Neospora* have been identified as one of the causes of abortion in livestock worldwide. Neosporosis causes mainly disease in dairy and beef cattle, whereas toxoplasmosis is a well-known zoonosis primarily causing disease in sheep and humans, but both are known to have a wide range of host species (Buxton *et al.*, 2007; Dubey & Lindsay, 1996; Dubey & Lindsay, 2006; Reid *et al.*, 2012). Sarcocystosis is known to cause miositis and encephalaitis in muscle and brain, and may lead to death and abortions (Dubey *et al.*, 2015a). These protozoan cause major economic losses and healthcare burden worldwide, decreasing production rates and increasing the costs of control and prevention measures, in farm industries (Adriana *et al.*, 2008; Buxton *et al.*, 2007; Dubey, 2003; Dubey *et al.*, 2006; Dubey & Lindsay, 2006; Goodswen *et al.*, 2013; Kaltungo & Musa, 2013; Odening *et al.*, 1996).

1.1.1. *Toxoplasma gondii*

Toxoplasma gondii was discovered in 1908 by Charles Nicolle and Louis Manceaux with its first diagnosis in tissues of a North African rodent, the gundi (*Ctenodactyle gundi*) (Nicolle & Manceaux, 1908), and by Alfonso Splendore who also discovered *T. gondii* in a rabbit (*Oryctolagus cuniculus*) in Brazil (Splendore, 1908). Both discoveries were initially thought to be *Leishmania*. The *Toxoplasma gondii* parasite was named by Nicolle and Manceaux (1908) based on its morphology, toxo meaning arc or bow, plasma meaning life and *gondii* after its original host animal, the gundi (*C. gundi*).

Since its discovery, numerous reports of *T. gondii*- like cases were reported in several warm-blooded animals including humans, making it arguably the most successful parasite worldwide (Ferguson, 2009). In the 1920s and 1930s, there were several reports that described the pathogenicity of *T. gondii* in humans as a result of congenital infections. In 1937, the first detailed study showed that *Toxoplasma* is an obligate intracellular parasite that is transmissible by intracranial, subcutaneous and intraperitoneal inoculation of brain homogenates (Sabin & Olitsky, 1937).

In 1957, ovine toxoplasmosis was first described by Hartley *et al.*, (1954). An outbreak of abortions in penned ewes was reported and *T. gondii* was identified in placental and foetal tissues, and *T. gondii* was associated with abortions in sheep (Hartley & Marshall, 1957). Transmission routes were initially unclear, and only two routes of transmission were known, the transmission via the consumption of undercooked meat containing tissue cysts or vertical transmission from mother to foetus. However, as sheep are herbivores and do not consume raw meat, another yet undiscovered transmission route was suggested. Hutchison (1965), showed that infected cats were able to shed an environmentally stable form (oocysts) of *T. gondii* via faeces. Subsequently, *Toxoplasma* abortion in sheep was connected with the feeding of grains containing contaminated cat faeces (Plant *et al.*, 1974). Oocysts were found to survive in temperate and moist environmental conditions, which could be the cause as to why ovine toxoplasmosis is a problem in most countries worldwide (Buxton & Rodger, 2007). It was not until the 1970s that the first description of the sexual development of *T. gondii* in the small intestine of cats was observed and felids were identified as the definitive host for this parasite (Dubey *et al.*, 1970; Frenkel *et al.*, 1970; Hutchison *et al.*, 1970). Nowadays, *Toxoplasma* is a well-known parasite that is able to infect and multiply in a wide range of warm-blooded animals, causing abortions following vertical transmission.

1.1.2. *Neospora caninum*

In Norway in 1984, a *T. gondii*-like parasite was described in a case of encephalomyelitis and myositis in the brain and spinal cord, that resulted in paralysis and early death of domestic puppies (Bjerkas *et al.*, 1984). Necropsy showed active inflammatory lesions in the brain, however, no antibodies to *T. gondii* were found in

the infected dogs, nor was it possible at the time to experimentally transmit it in mice and it hence was believed not to be *Toxoplasma*. *Neospora caninum* was initially confused and often misdiagnosed with *T. gondii*, due to its structural and morphological similarities. The unidentified species was formally recognised in 1988, as a cause of clinical disease in dogs in the USA named *Neospora caninum* (Dubey *et al.*, 1988a). The indirect fluorescent antibody test for the serologic diagnosis and the immunohistochemistry test for the identification of *Neospora* organisms in tissues were developed in 1988 and 1989, respectively (Dubey *et al.*, 1988b; Lindsay & Dubey, 1989).

In 1989, only a few years after the first identification of *Neospora* in dogs, cases of protozoan infections causing encephalitis in calves were reported, and *Neospora* was first associated with abortion outbreaks in cows in New Mexico (Thilsted & Dubey, 1989). Following the association of *Neospora* and abortions in cattle, experiments on a beagle and on Jersey cows demonstrated that *N. caninum* can be transmitted transplacentally in dogs and cattle (Dubey & Lindsay, 1989; Dubey *et al.*, 1992). McAllister *et al.* (1998), demonstrated that following the consumption of *Neospora* infected tissue cysts from mouse carcases, dogs shed oocysts resembling this protozoan parasite, and canids were thus identified as the definitive host of *N. caninum.*

The main differences between *T. gondii* and *N. caninum* pathophysiology is that in ovine toxoplasmosis, abortions mainly occur in the primary infection during pregnancy. In bovine neosporosis, the major route could be associated to the 'recrudescence of an established persistent *Neospora* infection' (Dubey *et al.*, 2006). Although *T. gondii* and *N. caninum* are both tissue-dwelling coccidia and share many structural and morphological similarities, they are biologically distinct. *Neospora* is quie specific in the intermediate host in comparison to *Toxoplasma* (Dubey, 2009). Moreover, *Toxoplasma* mainly causes abortions in sheep whereas *N. caninum* is a major cause of abortions in cattle. Additionally, the epidemiology of *N. caninum* is different in that it is efficient in vertical transmission whereas *Toxoplasma* is not (Dubey & Lindsay, 2006).

1.1.3. *Sarcocystis* spp.

Unlike the genera of *Toxoplasma* and *Neospora*, which only represent one and two species respectively, Sarcocystosis has more than 196 currently within the genus Sarcocystis (Dubey *et al.*, 2015a). *Sarcocystis* are very successful protozoan parasites as they infect high numbers of definitive and intermediate hosts, including all vertebrates, birds, reptiles, fish and mammals (Dubey, 2003; Kaltungo & Musa, 2013; Obijiaku *et al.*, 2013). Miescher (1948), reported the first case of Sarcocystosis as a 'milky white thread' in the skeletal muscle of a deer mouse caught in Switzerland. The zoological name of the mouse was not specified (Miescher, 1948). Subsequently, *Sarcocystis* was found in a house mouse (*Mus musculus*) and named *Sarcocystis muris*. It was believed to be the same species as the one previously discovered by Miescher (Dubey *et al.*, 2015a).

All *Sarcocystis* parasites have an obligatory two-host life cycle, asexual reproduction and sexual reproduction (Dubey & Lindsay, 2006). However, *Sarcocystis* are more specific for their intermediate hosts than for their definitive hosts (Dubey & Lindsay, 2006). For example, *Sarcocystis* species specifically found in cattle (*S. cruzi*, *S. hirsuta* and *S. hominis)* have shown to have different definitive hosts, canidae, felidae and primates, respectively (Dubey *et al.*, 1989b). Most species of *Sarcocystis* are normally non-pathogenic and do not cause illness in the definitive hosts, whereas pathogenicity is mainly manifested in intermediate hosts (Dubey & Lindsay, 2006).

Only a few species of *Sarcocystis* have shown pathogenicity and cause clinical signs in ruminants. The more pathogenic species of *Sarcocystis* can cause farm animals to abort and foetal growth can be slowed when infected during mid or late gestation of pregnancy (Barnett *et al.*, 1977; Dubey, 1981). Most infected animals are asymptomatic and show no obvious signs of disease but if lesions are present it causes eosinophilic myositis, an inflammatory condition of striated muscles (Dubey *et al.*, 2015a). Most infections are usually undetected but only discovered after microscopic examination (Dubey *et al.*, 2015a; Dubey & Lindsay, 2006).

1.2. Life cycles and transmission

Neospora, *Toxoplasma* and *Sarcocystis* all share a common heteroxenous lifecycle. The asexual reproduction takes place in the intermediate host, and the sexual reproduction occurs in the intestine of the definitive host: *Canids* for *N. caninum*, *felids* for *T. gondii* and various animals, including cats, dogs, foxes, raccoons and badgers for *Sarcocystis* s (Dubey, 2009; Dubey & Lindsay, 2006; Goodswen *et al.*, 2013; Reid *et al.*, 2012). In *T. gondii* and *N. caninum,* sexual reproduction results in the production of oocysts, which are shed into the environment with the faeces in an unsporulated form (Dubey & Lindsay, 2006; Shaapan, 2016) (**Figure 1.1** and **Figure 1.2).** In *Sarcocystis*, oocysts sporulate in the lamina propria, in the small intestine and are then released into the environment in the form of sporozoites (Dubey & Lindsay, 2006).

Within two to three days after excretion, *N. caninum* and *T. gondii* oocysts sporulate with each oocyst containing two sporocysts, and each sporocyst contain four sporozoites (Dubey *et al.*, 2002; Dubey & Lindsay, 2006; Reichel *et al.*, 2007). Once the intermediate or definitive host ingest the sporulated oocysts these excyst and sporozoites are released into the small intestine (Goodswen *et al.*, 2013). They subsequently invade the gut wall and transform into tachyzoites (Goodswen *et al.*, 2013). Tachyzoites replicate rapidly by asexual endodyogeny, invading various nucleated cells such as smooth cardiac and skeletal muscle cells, hepatocytes cells, fibroblasts and macrophages, then spread via the blood and lymphatic system before differentiating into bradyzoites forming tissue cysts in the central nervous system (CNS) and skeletal muscle cells (Dubey *et al.*, 2002). Bradyzoites slowly replicate asexually by endodyogeny, penetrate the intestinal epithelium and start sexual reproduction giving rise to schizonts (Dubey & Lindsay, 1990). Merozoites released from those schizonts form into male and female gamonts, fertilize to form a macrogamont and produce oocysts (Dubey & Lindsay, 1990).

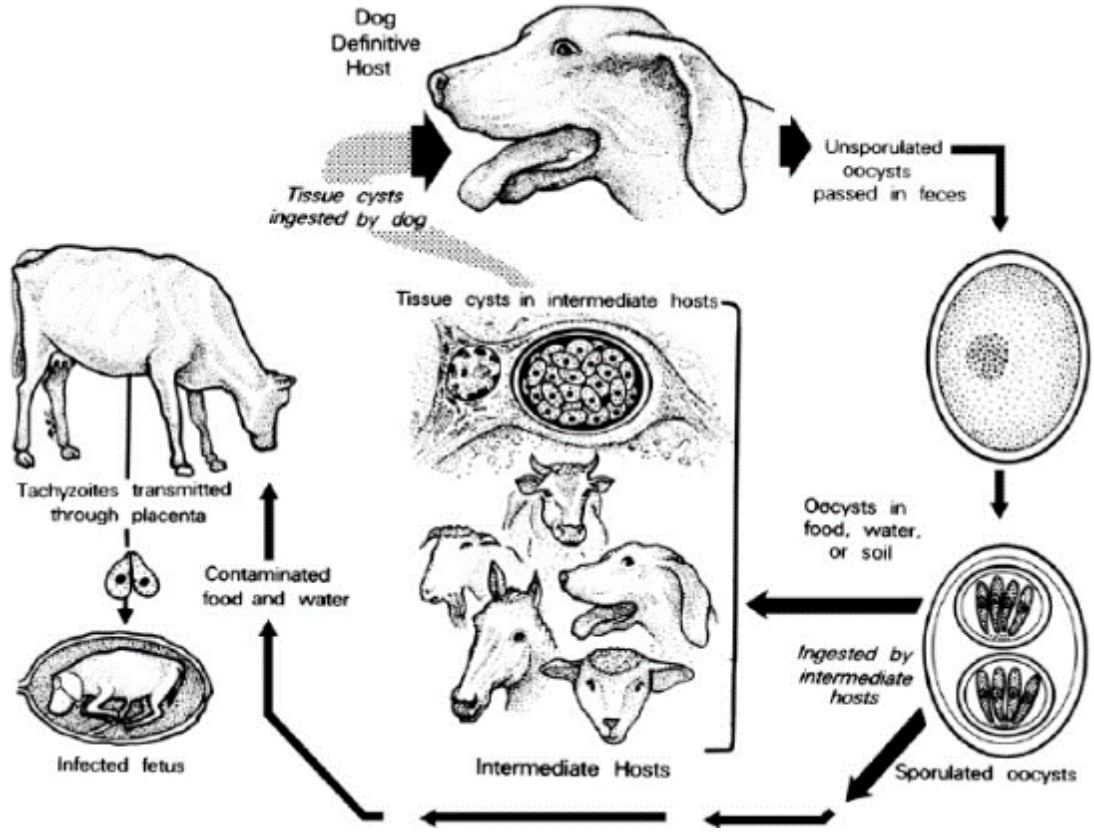


FIGURE 1.1. THE LIFE CYCLE OF *NEOSPORA* SHOWING VERTICAL AND HORIZONTAL TRANSMISSION STAGES. THE IMAGE IS TAKEN FROM DUBEY AND LINDSAY (2006).

Tissue cysts can persist for the life of the host and when eaten by an intermediate or definite host can transmit the parasite (Dubey *et al.*, 2004) (**Figure 1.1** and **Figure 1.2).** In *Sarcocystis* spp. after ingestion of sporocysts, sporozoites are released forming the first-generation schizonts in endothelial cells of the arteries which give rise to merozoites (**Figure 1.3**). Second-generation schizonts are formed in capillaries from 19 to 46 days (Dubey & Lindsay, 2006). The merozoites penetrate the host cells and undergo repeated division producing sarcocysts containing bradyzoites (Dubey & Lindsay, 2006). Following ingestion bradyzoites transform into male (micro) and female (macro) gamonts in the intestinal epithelium producing oocysts, undergo sporogony creating two sporocysts and undergo lysis before releasing the sporocysts (Dubey *et al.*, 2015c).

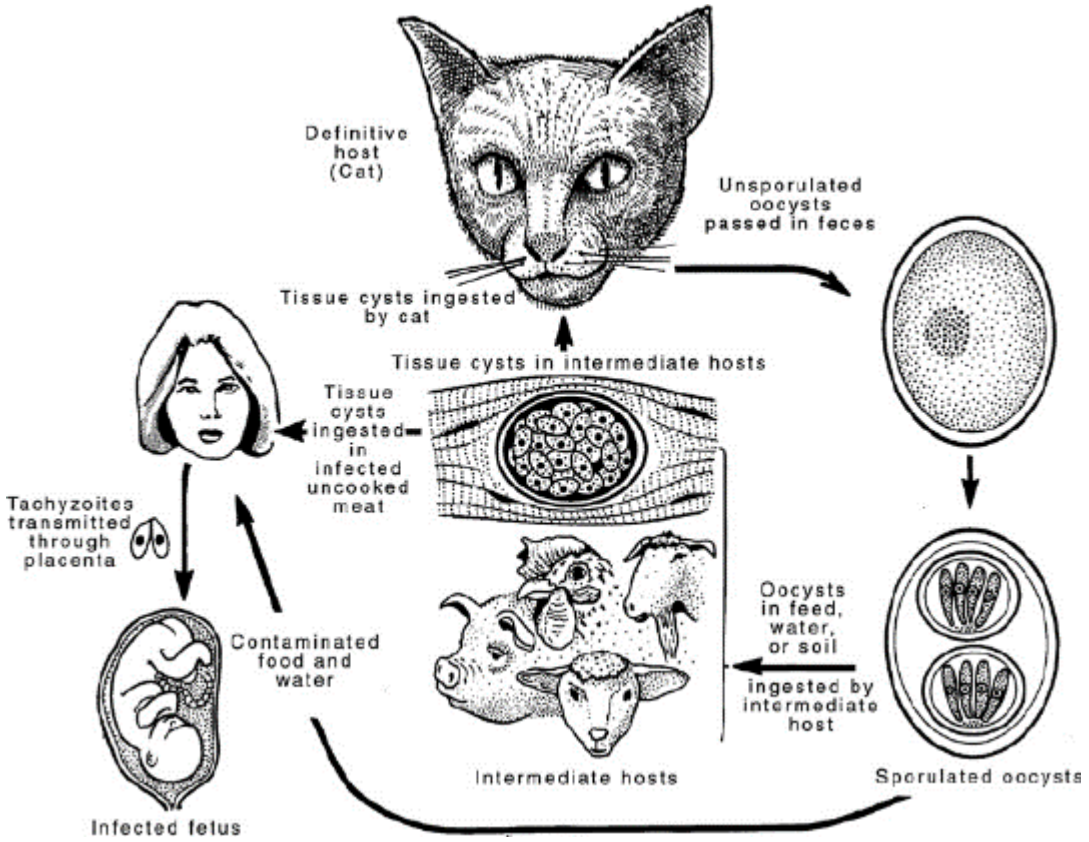


FIGURE 1.2. THE LIFE CYCLE OF *TOXOPLASMA* SHOWING VERTICAL AND HORIZONTAL TRANSMISSION STAGES. THE IMAGE IS TAKEN FROM DUBEY AND LINDSAY (2006).

The life cycles of all three protozoan parasites are similar and share common transmission routes of infectious stages in their life cycles. Various specialised protozoan organelles, such as surface antigens and specialised apical secretory organelles help in the transmission of the parasites (Reid *et al.*, 2012).

There are three infective stages of the parasites; tachyzoites, sporozoites (oocysts) and bradyzoites (tissue cysts), which are transmitted as follows: either due to ingestion of oocysts or sporocysts (containing sporozoites) in faeces / soil, or by consumption of animal tissues containing tissue cysts / tachyzoites / sarcocysts (with slowly proliferating bradyzoites) or transmission of tachyzoites from mother to foetus (Dubey *et al.*, 2002; Innes, 2010; Shaapan, 2016) (**Figure 1.1, Figure 1.2** and **Figure 1.3**).

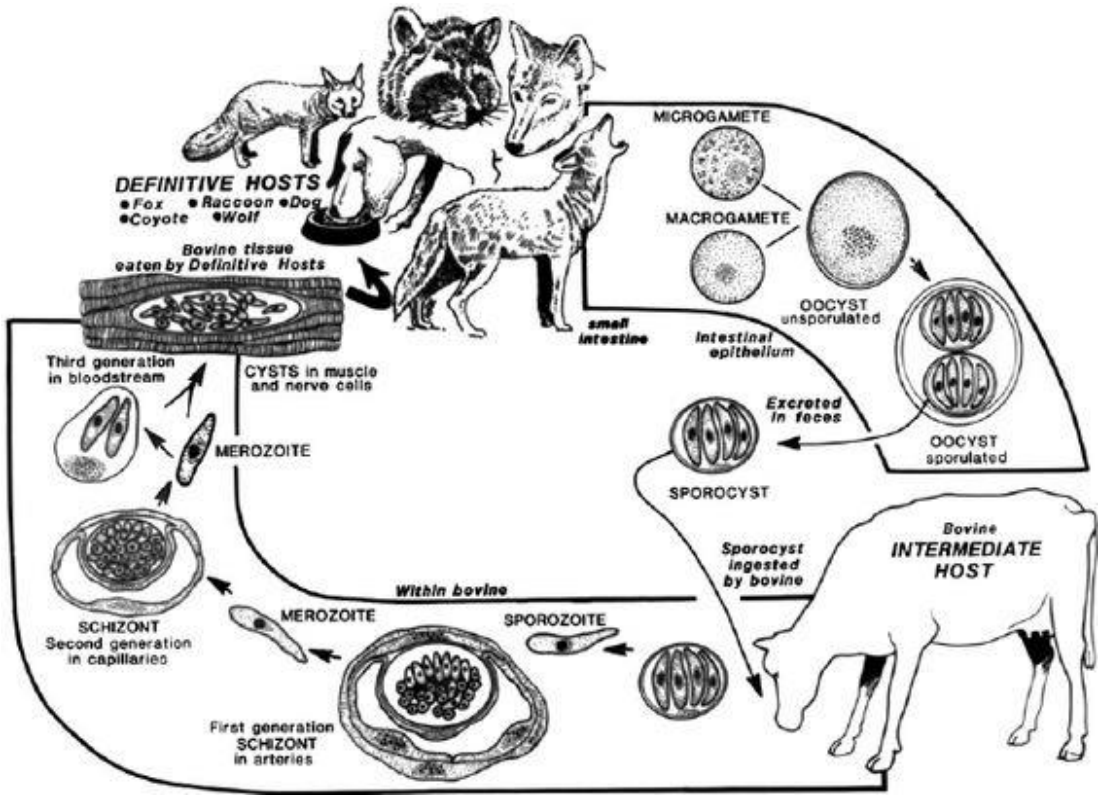


FIGURE 1.3. THE LIFE CYCLE OF *SARCOCYSTIS* SPP. SHOWING VERTICAL TRANSMISSION STAGES. THE IMAGE IS TAKEN FROM DUBEY AND LINDSAY (2006).

Toxoplasma and *Neospora* have been shown to be transmitted vertically (in utero), where rapidly proliferating tachyzoites are transferred transplacentally from mother to foetus often causing abortions or stillbirths (Dubey *et al.*, 2015c; Dubey *et al.*, 1990; Dubey & Lindsay, 2006; Goodswen *et al.*, 2013). For *Neospora*, it is known that transplacental transmission can occur either by exogenous or endogenous transplacental infection (Dubey *et al.*, 1990).

Exogenous infection happens when the dam acquires the infection by ingesting oocysts during pregnancy (Trees & Williams, 2005). In endogenous transmission the dam was infected with the parasite prior to pregnancy, and due to re-activation of bradyzoites into tachyzoites, the foetus is infected (Trees & Williams, 2005). *Neospora* and *Toxoplasma* DNA has been detected in milk and colostrum making transmission of lactogenic routes possible (Davison *et al.*, 2001; Moskwa *et al.*, 2007). The study by Davison *et al.*, (2001) has shown that transmission of the parasite to neonatal calves was possible when ingesting milk that was experimentally spiked with tachyzoites. Abortions may also occur during *Sarcocystis* infections when animals become infected

with a pathogenic species (Dubey *et al.*, 2015a). The mechanism by which *Sarcocystis* induce abortions is still unknown (Pescador *et al.*, 2007b). Free merozoites and schizonts, commonly associated with lesions, have been found in placentas from aborted cattle (Dubey & Bergeron, 1982; Fayer & Dubey, 1988).

1.3. Similarities and differences: Gene targets

The small differences in the biology of each parasite can provide an opportunity to identify the mechanisms for the basis of host specificity, pathogenesis and zoonotic potential. Each protozoan parasite differs in the molecular determinant of host specificity, and varies at the genetic level (i.e. genes and proteins). The genome of *S. neurona* (127Mbp) has shown to be more than twice the size of the genome of *Toxoplasma* (61Mbp) and *Neospora* (63Mbp) (Blazejewski *et al.*, 2015; Reid *et al.*, 2012). Differences in particular among the group of genes that interact with the host, such as host cell invasion machinery and evasion from the host immune system common to all Apicomplexan, have been observed (Blazejewski *et al.*, 2015; Reid *et al.*, 2012).

The genetic determinants of host specificity such as surface antigen gene (SAG) families, SAG-1 related sequences (SRS) and apical secretory organelles (i.e. rhoptries (ROP), micronemes (MIC) and dense granule genes (GRA) are important factors for parasite invasion, host cell attachment, host-parasite interactions and parasite survival in the host (Reid *et al.*, 2012). These protozoan parasites attach to host cells via highly abundant SAG family proteins (i.e. SAG1), after which adhesins are released by micronemes and drive the actin-myosin motor for invasion into the host cell (Jung *et al.*, 2004; Keeley & Soldati, 2004). The SRS proteins exist as a developmentally regulated superfamily that promotes host cell attachment and control host immunity to regulate virulence and growth (Blazejewski *et al.*, 2015). Rhoptry neck proteins are next released, forming a moving junction that allows parasites to enter the cell and form a parasitophorous vacuole (PV) (Alexander *et al.*, 2005; Blader & Saeij, 2009; Talevich & Kannan, 2013). Rhoptries release kinases that modulate the host cell function and interact with host defences, such as ROP18 which inactivates host immunity-related GTPases needed to rupture the PV and kill the parasite (Fentress *et al.*, 2010). Dense granules known to be secretory organelles secrete vesicles into the

lumen of the PV, which are needed to establish correct functioning of the vacuole (Dubremetz *et al.*, 1993). This organelle-specific machinery is comprised of various gene products that are either up or down-regulated depending on stage-specific expression (Dubey & Lindsay, 1996; Hemphill *et al.*, 2006; Reid *et al.*, 2012). Each protozoan possesses unique gene-specific differences that may allow the identification of specific parasites.

A number of proteins anchored to the surface membrane with glycosylphosphatidylinositol (GPI)-anchored antigens of *T. gondii, N. caninum and Sarcocystis* were identified (Dubey *et al.*, 1990; Hehl *et al.*, 1997; Nagel & Boothroyd, 1989; Reid *et al.*, 2012). The SAG surface proteins are known to be relatively abundant and immunodominant, and are members of SAG1 and SAG2 families (Lekutis *et al.*, 2000; Nagel & Boothroyd, 1989). The major surface antigens of *T. gondii*, TgSAG1 (P30) (Nagel & Boothroyd, 1989), TgSAG3 (P43) and TgSAG2A (P22) (Cesbron-Delauw, 1995; Prince *et al.*, 1990) and SAG-1 related sequences (SRS), such as TgSRS2 have been shown to be specifically expressed the tachyzoite and/or the bradyzoite stages. Similar homologues in *Neospora*, NcSAG1 (P29) and NcSRS2 (P43) and in *Sarcocystis* neurona, SnSAG1, SnSAG2, SnSAG3, and SnSAG4, were identified (Gottstein *et al.*, 1998; Hemphill & Gottstein, 2006; Howe *et al.*, 2005; Kasper *et al.*, 1984; Lekutis *et al.*, 2001).

The surface antigens encoded by the SRS gene family of *N. caninum* show considerable differences compared to those of *T. gondii*, and were shown to be substantially expanded in *Neospora* compared to *Toxoplasma* (Reid et al., 2012). However, *T. gondii* was shown to express a greater number of the SRS repertoire during the tachyzoites stage: 55 compared to 25 in *N. caninum*, whereas *S. neurona* showed a more restricted set of SRS-encoding genes with only 23 (Blazejewski *et al.*, 2015; Hemphill & Gottstein, 2006; Reid *et al.*, 2012). Each set of genes are distinctively expressed for each parasite. During the tachyzoite to bradyzoite stage conversion., it was shown that NcSAG1 was down-regulated and various bradyzoites-stage specific genes such as NcSAG4, NcBSR4, NcMAD1, NcSRS13, were up-regulated (Reid *et al.*, 2012).

A greater number of bradyzoite-specific genes were expressed in *N. caninum* compared to *T. gondii*: 36 versus 25 genes, respectively (Fernandez-Garcia *et al.*, 2006; Guionaud *et al.*, 2010; Reid *et al.*, 2012; Risco-Castillo *et al.*, 2007). Similar orthologues, TgSAG1 and TgSRS2, were down-regulated in *T. gondii* and various bradyzoites genes, such as TgSRS9 and TgSAG4 were up-regulated (Blader & Saeij, 2009). *Sarcocystis* spp. has also been known to exhibit various levels of similarities to the SAG gene family of *T. gondii*. As yet only *S. neurona* SAG orthologues, such as SnSAG1, SnSAG2, SnSAG3, and SnSAG4 have been identified, and similarly to *Toxoplasma* and *Neospora* the SAG of *S. neurona* are expressed during the invasion and evasion stage (Ellison *et al.*, 2002; Howe *et al.*, 2008; Howe *et al.*, 2005). The *Sarcocystis* SAG proteins were identified based on their homology to the extensive family of GPI-anchored surface antigens, in related tissue coccidian parasite *T. gondii* (Lekutis *et al.*, 2001) and *N. caninum* (Hemphill & Gottstein, 1996). SnSAG1 and SnSAG2 are major immune-dominant antigens, and play a major role in host invasion and evasion of the immune response (Howe *et al.*, 2005). However, only limited information is available on genes for *Sarcocystis* species.

Micronemes and their genes (MIC genes) play a crucial role in mediating the gliding motility during host cell invasion (Jung *et al.*, 2004). Some genes have been shown to be specific to *N. caninum* (MIC26 and MIC19), whereas others are solely expressed in *T. gondii* (i.e. MIC7, MIC8, MIC20, MIC15) (Blazejewski *et al.*, 2015; Keeley & Soldati, 2004; Reid *et al.*, 2012). The genome of *S. neurona* has also reported orthologues of *T. gondii* MICs (Blazejewski *et al.*, 2015). Differences in organelle-specific gene expression were also observed in rhoptries between *Neospora* (i.e. ROP1B, ROP55), *Toxoplasma* (i.e. ROP18 and ROP8) and *S. neurona* (i.e.ROP20, ROP26) (Blazejewski *et al.*, 2015; Reid *et al.*, 2012). Rhoptry kinase genes (ROP) (i.e. ROP18, ROP16 and ROP5) have been associated with virulence in *T. gondii*, yet homologues in *Neospora* have been found to differ (Reid *et al.*, 2012). For example, TgROP16 has been shown to interfere with host signalling and was highly expressed in *T. gondii* tachyzoites, whereas the orthologue of *Neospora* (NcROP16) was not expressed (Bahl *et al.*, 2010). Various ROP genes were missing in the *N. caninum* repertoire, such as ROP2A, ROP2B and ROP5 (Reid *et al.*, 2012). ROP orthologues specific to *S. neurona* were identified (i.e. SnROPKs, SnROP34, ROP21, ROP27) and

are expressed during the merozoites stage (Blazejewski *et al.*, 2015). Furthermore, dense granule genes (GRA) also demonstrated similar homologues between *Neospora* and *Toxoplasma*, such as GRA7 and GRA6, and were found to be associated with the PV membrane and the vacuolar network (Jacobs *et al.*, 1998; Lecordier *et al.*, 1995; Mercier *et al.*, 2002).

On the other hand, some differences in GRA genes were found. GRA11 and GRA12 were found to be absent from *N. caninum* and thus specific to *T. gondii* (Reid *et al.*, 2012). Only two dense-granule protein (GRA10 and GRA12) homologous of *T. gondii* were identified in *S. neurona* (Blazejewski *et al.*, 2015). Only a limited repertoire of GRA proteins was found in the genome of *Sarcocystis*, as they may not require them during merozoite replication since it replicated in the host cytosol, and it is not contained within a PV like *T. gondii* and *N. caninum* (Blazejewski *et al.*, 2015).

The online database ToxoDB (http://toxodb.org/toxo/) and NCBI (https://www.ncbi.nlm.nih.gov/) provides detailed information on gene expression and proteins available for *N. caninum*, *T. gondii* and *Sarcocystis* spp. The databases can be used to search for specific genes. Numerous protozoan-specific gene differences between *T. gondii*, *N. caninum* and *Sarcocystis* spp., could be used to produce genus-specific recombinant proteins for the development of specific diagnostic tests. This is due to their species-specific nature (*T. gondii*, *N. caninum* and *Sarcocystis* spp.), and their stage-specific gene expression pattern exhibited between the various transmission stages (i.e. tachyzoites and bradyzoites).

1.4. Disease in ruminants

As previously mentioned, ruminants (cattle, sheep, goats, and water buffalo) are known to play a crucial part as the intermediate hosts of the protozoan parasites *Neospora*, *Toxoplasma* and *Sarcocystis* spp. Infections of the host with each protozoan parasite depend on many factors, such as the housing and feeding of the animal. Prevalence of infections depends on the presence of definitive hosts, such as cats and dogs, to transmit the parasites. Ruminant livestock are known to become infected horizontally by ingestion of environmental oocysts shed from cats / dogs, by grazing contaminated vegetation, consuming contaminated feed or drinking contaminated

water (Almeria & Lopez-Gatius, 2013; Dubey & Lindsay, 2006; Innes *et al.*, 2009). Vertical transmission may also occur by transplacental transmission of tachyzoites from mother to foetus in *Neospora* or *Toxoplasma* infected animals (Dubey *et al.*, 2015a; Dubey & Lindsay, 2006; Dubey *et al.*, 1992; Innes *et al.*, 2009). As ruminants are not carnivorous, ingestion of tissue cysts from contaminated meat are unlikely. However, infected carcasses of small rodents that may have dried up in the feed could be accidentally ingested making ingestions of tissue cysts possible. Mixed protozoal infections in both large and small ruminants are possible, making identification of individual protozoan species in the same host, and determining which species was the cause of the disease, particularly difficult. The following section will cover details of *N. caninum, T. gondii* and *Sarcocystis* spp. infections in cattle, sheep, buffaloes and goats.

1.4.1. Cattle (*Bos taurus*)

Neospora caninum is generally viewed as the principal abortifacient agent in cattle and can cause abortions in both dairy and beef cattle worldwide with reported national sera prevalence rates of 16-76% and 41-61%, respectively in the USA (Bartels *et al.*, 2006; Dubey *et al.*, 2006; Dubey *et al.*, 2007; Thilsted & Dubey, 1989). Cows abort during any age from 3 months of gestation to term, with most abortions occurring between the 5^{th} and 7^{th} months of gestation (Almeria & Lopez-Gatius, 2013; Dubey, 2005; Dubey & Lindsay, 2006; Dubey *et al.*, 2007).

Neospora infections may lead to several different clinical manifestations. Foetuses may die *in utero*, become reabsorbed, aborted, stillborn or born deformed or mummified, calves may be born underweight or with neurological impairment or are born clinically normal but infected (Dubey, 2005; Dubey *et al.*, 2006; Mazuz *et al.*, 2014; McAllister, 2016). Transmission of *Neospora* from the dam to offspring is highly efficient, and a vertical transmission rates were reported to be as high as 95% (118/124) in Friesian dairy herds in a study in the UK (Davison *et al.*, 1999), 93% (14/15) in Friesian dairy cattle herds in a Germany (Schares *et al.*, 1998), and 81% (93/115) in a study in Californian dairy herds (Paré *et al.*, 1996). Congenitally infected calves can remain persistently infected for life, and might undergo abortions in subsequent pregnancies (Almeria & Lopez-Gatius, 2013; Pabon *et al.*, 2007). In some

herds, up to 33% of cows can abort over a period of a few months (Dubey & Schares, 2006; Hassig *et al.*, 2003; McAllister *et al.*, 2000).

Cows with *N. caninum* antibodies (seropositive) are more likely to abort than seronegative cows (Dubey & Lindsay, 2006; Nogareda *et al.*, 2007). Previous studies have shown that the abortion risk is up to 23 times higher in seropositive than in seronegative cows (Nogareda *et al.*, 2007; Reichel *et al.*, 2013). Most beef and dairy calves born to *Neospora* seropositive dams remain clinically healthy, however even though they do not exhibit any abnormalities, they could be chronically infected and are thus able to transmit the parasite transplacentally to their progeny (Dubey, 2003; Dubey & Lindsay, 2006; Mazuz *et al.*, 2014). In several studies, transplacental transmission in persistently infected parous cows were associated with an significant increase of serological antibodies against *Neospora* (Guy *et al.*, 2001; Lopez-Gatius *et al.*, 2005). The consequences of infection in cows may vary depending on various factors, such as timing of the infection, the duration of parasitemia in the pregnant cow, as well as the maternal and foetal immune response (Innes, 2007; Innes *et al.*, 2005; Mazuz *et al.*, 2014).

As most cows infected with *N. caninum* do not exhibit any clinical signs except abortions, the best prevention and control of neosporosis is an early diagnosis and allowing the implementation of preventative measures to control the disease in a herd. Neosporosis in cattle has been shown to occur worldwide, and a recent analysis estimated the cost of neosporosis to be $546 million to the dairy industry and $111 million to the beef industry, per annum in the USA (Reichel *et al.*, 2013). An overview of the prevalence of neosporosis using data from ten different countries reports that dairy cattle show a 50% higher infection rate than beef cattle (Reichel *et al.*, 2013). Even though abortions in cattle due to protozoan infections are associated with *Neospora*, other protozoan parasites, such as *T. gondii*, have also been previously reported in cattle (Dubey, 2005).

Toxoplasma is known to infect many warm-blooded animals, including cattle. Initially, natural infections of *Toxoplasma* in cattle were not associated with any clinical signs, and various studies have shown that toxoplasmosis does not cause abortions in cattle under natural conditions, but may still be present in their tissues (Dubey, 1986;

Koestner & Cole, 1961). Although bovines can be infected with *T. gondii* oocysts, it was shown by Dubey (1983) that the parasites were eliminated to an undetectable level within a few weeks post-infection (Burrells *et al.*, 2013). In two experimentally infected calves examined ten days after infection, lesions in the central nervous system were observed, and *T. gondii* parasite was isolated from several tissues from the infected calves (Koestner & Cole, 1961). Various studies have observed that *Toxoplasma* DNA can be isolated and amplified from aborted bovine foetuses (Amdouni *et al.*, 2017; Canada *et al.*, 2002; Dubey & Schares, 2006). For example, in the study by Gottstein *et al.*, (1998), *T. gondii* DNA was detected in 4/83 (5 %) of aborted bovine foetuses, and *T. gondii* was confirmed by serology in 2 cattle. Another study by Ellis (1998), tested 40 foetuses of which 16 were shown positive for *Neospora* DNA and 2 samples were shown positive for *T. gondii* DNA. Other studies have shown the detection of *T. gondii* antibodies by enzyme-linked immunosorbent assay (ELISA), as it was reported in the study by Opsteegh *et al.*, (2011b), where 22.7% (145 / 638) of cattle samples were seropositive for *T. gondii*. Moreover, mixed infection of *Neospora* and *Toxoplasma* can also be seen. For example, in a study carried out in the northern regions of Brazil by Da Silva *et al.*, (2017), 10.6 % of cattle sampled (n= 500) showed specific antibodies to both *T. gondii* and *N. caninum* by indirect fluorescent antibody test.

Other studies have shown that in *T. gondii* experimentally infected cattle, abortions are possible (Canada *et al.*, 2002; Wiengcharoen *et al.*, 2011). In the study by Wiengcharoen *et al.*, (2011), it is reported that two out of four heifers aborted when experimentally infected with *T. gondii*. The same study found *T. gondii* tachyzoites in various organs of the dams that had aborted. The study observed that pregnant cattle infected with *T. gondii* showed clinical disease and resulted in abortion, hence indicating that vertical transmission in cattle is possible when infected with *Toxoplasma* (Wiengcharoen *et al.*, 2011). Similar results were observed in cattle inoculated with a virulent strain of *T. gondii*, which had resulted in clinical disease and abortions (Dubey, 1986; Dubey & Fayer, 1986; Munday, 1978; Stalheim *et al.*, 1980). Both *Neospora* and *Toxoplasma* can be vertically transmitted from mother to offspring in cattle, yet the vertical transmission of *T. gondii* mainly occurs when the dam acquires an infection during pregnancy, whilst *N. caninum* can be transmitted to the

offspring, if dams are infected either before or during pregnancy (Wiengcharoen *et al.*, 2011).

Natural *T. gondii* infections in cattle are usually asymptomatic, as the level of infection is not severe enough to cause disease and because cattle seem to have high natural resistance to *T. gondii* (Burrells *et al.*, 2018; Costa *et al.*, 2011; Wiengcharoen *et al.*, 2011). Yet, the potential of abortions in cattle could be possible, as shown from experimental studies. The prevalence of *T. gondii* in cattle seems to vary between different studies in different countries. The prevalence can range from 83.3% (n= 504) from 72 herds in southern Spain (Garcia-Bocanegra *et al.*, 2013), 71.0% (n= 1420) from 50 cattle herds in Brazil (Santos *et al.*, 2009), and 45.6% (n= 406) from calves, heiferes, bulls and cows from different herds in Switzerland (Berger-Schoch *et al.*, 2011), to 37.9% (n= 127) from 190 different cattle herds in Poland (Holec-Gasior *et al.*, 2013) and 19.3% (29/150) from cattle's slaughtered for meat consumption in a study in north-west Tunisia (Amdouni *et al.*, 2017).

Furthermore, many studies have reported a variety of *Sarcocystis* spp. in cattle (Dubey *et al.*, 2015a; Dubey *et al.*, 2014; Dubey *et al.*, 1989a). The clinical severity of infection and susceptibility to the infection depend on the dose of infection, as well as if an animal is under a situation that could modulate its immune response (i.e. pregnant, lactating and poor nutrition). Pregnant animals may abort, or growth may be slowed or arrested (Dubey *et al.*, 2015a). Transmission of *Sarcocystis* has been mainly confirmed by horizontal routes. Vertical transmission of *S. cruzi* has been documented in experimental various studies. For example, in the study by More *et al.*, (2009), vertical transmission was observedin only 1.7 % (3/173) of cases, and showed that 3 dams delivered seropositive calves. However, it has rarely been observed in natural infections (Dubey & Bergeron, 1982; Lopes *et al.*, 2005; More *et al.*, 2009). Clinical signs are generally observed during the schizogonic cycle in the blood vessels (acute phase) and are fever, anorexia or anaemia, and some animals even die (Dubey & Lindsay, 2006). As previously mentioned *Sarcocystis* spp. generally do not tend to cause clinical signs in the definitive host, and most species only show pathogenicity in the intermediate hosts (Dubey *et al.*, 2015a; Dubey & Lindsay, 2006).

Cattle are known to be intermediate hosts for various *Sarcocystis* spp., namely *S. cruzi, S. hirsuta, S. rommeli, S. hominis, S. sinensis, S. bovini*, *S. bovifelis*, and *S. heydorni* (Dubey *et al.*, 2015a; Dubey & Lindsay, 2006; Dubey *et al.*, 2016; Gjerde, 2016a; Gjerde, 2016b; Yang *et al.*, 2001a). The *Sarcocystis* species *S. cruzi* (for which the dog is a definitive host) is more pathogenic in cattle than *S. hirsuta*, and *S. hominis* (for which have cats and primates as their definitive hosts) (Dubey *et al.*, 2015a; Dubey & Lindsay, 2006; Dubey *et al.*, 1989b). *Sarcocystis hominis* is a known zoonotic, as it has been shown to cause sarcocystosis in humans (Fayer *et al.*, 2015). High levels of *Sarcocystis* cysts in muscles from adult cattle have been reported in most regions of the world, ranging up to 100% (Ahmed *et al.*, 2016; Amairia *et al.*, 2016; De Bosschere & Ducatelle, 2001; Mirzaei & Rezaei, 2016; More *et al.*, 2008; Nourollahi Fard *et al.*, 2009; Pena *et al.*, 2001; Vangeel *et al.*, 2013; Vangeel *et al.*, 2012). High burden of *Sarcocystis* tissue cysts in samples for human consumption could be problematic as the meat will be discarded following microscopic testing, and as certain *Sarcocytis* species can be transmitted to humans causing sarcocystosis. In the study by Pena *et al.*, (2001), 50 bovine samples obtained from various food establishments in Sao Paulo were examined for *Sarcocystis* spp., using light and electron microscopy, and based on cyst wall strucutre species were identified as *S. hominis* (94 %), *S. hirsuta* (70 %), and *S. cruzi* (92 %), mostly as mixed infections.

1.4.2. Sheep (*Ovis aries*)

Toxoplasma infections in small ruminants such as sheep and goats are common and are the major cause of abortions worldwide resulting in major economic losses in sheep and goat industries (Buxton *et al.*, 2007; Dubey, 2010; Innes, 2010; Innes *et al.*, 2009). Toxoplasmosis is estimated to be involved in approximately 15 - 30% of abortions in sheep, and in the UK alone the cost of abortion due to *Toxoplasma* was estimated to be around £12 million in 2011, due to loss of production, cost of treatment, control and monitoring of the disease (Bennett, 2003; Bennett & IJpelaar, 2005; Hutchinson *et al.*, 2011). Clinical ovine toxoplasmosis generally occurs following primary infection with sporulated oocysts or through transplacental transmission of the parasite from mother to foetus (Buxton *et al.*, 2007; McColgan *et al.*, 1988). Morley *et al.*, (2008) and Williams *et al.* (2005) suggested that endogenous transplacental transmission resulting

from the activation of the quiescent bradyzoite stage during pregnancy is efficient and has shown to occur in persistently infected ewes but was shown to be infrequent. This route of infection was initially thought to be in-frequent, yet more recently has been shown to pose a significant risk in sheep (Buxton *et al.*, 2007; Buxton & Rodger, 2007, Klauk *et al*., 2016).

Most animals that are reported to be seropositive for *Toxoplasma* infections are generally asymptomatic. However, clinical signs become obvious when *T. gondii* primary infections occur in pregnant ewes, and signs can be present from mid-pregnancy to full-term (Buxton & Rodger, 2007; Esteban-Redondo & Innes, 1997; Shaapan, 2016). Infections lead to abortions, embryonic death, stillbirths, mummified foetus and neonatal death (Buxton *et al.*, 2007; Dubey & Lindsay, 2006). The clinical outcomes of transplacental transmission of *T. gondii* are determined by the stage of pregnancy (early/late term of gestation) the ewes are infected. Infections earlier in gestation when the foetus has an immature immune system, can lead to foetal death or abortions (Buxton *et al.*, 2007). Infections of *T. gondii* during mid-gestation can result in stillbirth, mummified foetus and weak lambs, whereas infections that occur during the final term of gestation may result in the lambs being born clinically normal but persistently infected (Buxton *et al.*, 2007).

Once sheep or goat become infected with *Toxoplasma*, specific IgG antibodies against *T. gondii* remain for a lifetime (Dubey & Jones, 2008). This can be verified as a higher prevalence of *T. gondii* can be observed in older animals. In a study by Katzer *et al*., (2011), 56% (1619/3333) from 125 Scottish sheep flocks were found positive for *T. gondii* and at least one animal in every flock was tested positive. This study had found that seropositivity is higher in animals (73.8%) over the age of 6 compared to animals of less than one 1 year old (37.3%). A high seroprevalence in older animals could be due to longer timespan that increased the chances of ingestion of oocysts from the environment (Dubey & Jones, 2008; Katzer *et al.*, 2011).

The importance of *T. gondii* infections in sheep has been reported in different countries and can been seen from the prevalence data reported in various studies. In the study by Diakoua *et al.*, (2013), specific *T. gondii* antibodies were detected in 53.7% (246/458) of sheep from 50 mixed flocks from various regions in Greece. Another

study collected blood samples from 547 clinically normal adult sheep from nine different farms in the Czech Republic and reported that 59% (325/547) sheep showed antibodies against *T. gondii* (Bártová *et al.*, 2009). In the study by Almeria *et al.*, (2018), a high seroprevalence of 41.2% (80/194) in sheep flocks was reported from 29 farms from the Mediterranean ecosystem.

Neospora caninum is mainly responsible for abortions in cattle. However, neosporosis was also shown to cause reproductive failures and abortions in sheep and goats (Dubey & Schares, 2011). Clinical signs in ovine neosporosis are similar to those present in bovine neosporosis (Dubey & Lindsay, 1990; Dubey & Lindsay, 2006; Moreno *et al.*, 2012). The study by Gonzalez-Warleta *et al.*, (2014), showed that *N. caninum* infections were the main cause of reproductive failure in sheep flocks from a farm in the province of Lugo in Spain and showed that parasite DNA and antibodies for *N. caninum* and histological lesions were found in aborted foetuses, stillbirths and neonatal lambs. These results showed that, 59.3% (54/91) of lambing ewes in 2011 and 72.1% (31/43) of lambing ewes in 2012 were seropositive for *N. caninum*, and had significantly lower percentage of lambs compared to *T. gondii* positive ewes (Gonzalez-Warleta *et al.*, 2014).

Low productivity in livestock is often caused by poor disease control. Differentiation between protozoan infections is key to adopt correct control strategies. Initially, natural abortion cases associated with *Neospora* in sheep and goats have not frequently been reported potentially, probably due to misdiagnosis as *Toxoplasma* (Dubey, 2003; Givens & Marley, 2008; Moreno *et al.*, 2012). Experimental studies have shown that pregnant sheep are very susceptible to *N. caninum* infections causing pathology similar to cattle (Buxton *et al.*, 1997; Buxton *et al.*, 1998; Dubey & Lindsay, 1990; Hassig *et al.*, 2003; McAllister *et al.*, 1996a). The study by Moreno *et al.*, (2012) found that 10.8% (8/74) of ovine foetuses submitted to the diagnostic Service of Exopol in Spain, had characteristic lesions suggestive of protozoal infections, and that the lesions were more frequently associated with the presence of *N. caninum* (62.5%) than *T. gondii* (37.5%). This study showed that *N. caninum* played a significant role in abortions cases in small ruminant comparable to *T. gondii* infections (Moreno *et al.*, 2012). Moreover, Arranz-Solis *et al.*, (2015), demonstrated that the severity of *Neospora* infections varied based on gestational periods, and that 100% of abortions from

experimentally infected Churra sheep (n= 50) took place during the initial gestation period, whereas lambs were all born prematurely and weak when infection occurred at the third term of gestation.

After *Neospora* infections, lesions of neosporosis found in the foetus and placenta of sheep resemble those seen in bovine neosporosis and ovine toxoplasmosis (Buxton *et al.*, 1997; Dubey & Lindsay, 1990; Hassig *et al.*, 2003; McAllister *et al.*, 1996a; Moreno *et al.*, 2012). Like *toxoplasmosis* in sheep, *neosporosis* in sheep showed a higher seroprevalence in older animals compared to younger animals. This was also shown in the study by Tembue *et al.*, (2011) who demonstrated a significantly higher *Neospora* infection in older animals from 23 different properties in Brazil and showed that sheep older than 4 years old had a higher *N. caninum* prevalence (80.0%) when compared to sheep up till one year old (41.7%) (Tembue *et al.*, 2011).

Sheep can also be infected with several different species of *Sarcocystis*, and which are normally identified during microscopic examination. Carcasses showing marcoscopic cysts are normally discarded during meat inspections causing major production losses (Martínez-Navalón *et al.*, 2012). The cost of *Sarcocystis* infections in small ruminant was estimated to be around 20 million euros a year in a study in Spain from 145 farms (Martínez-Navalón *et al.*, 2012). Among the previously reported species of *Sarcocystis*, *S. tenella* and *S. arietianis* (transmitted by canids) are frequently identifiable by microscopic cysts morphology, and *S. gigantean* and *S. medusiformis* (transmitted by cats) are identified by the observation of macroscopic cysts in the muscle tissue (Dubey *et al.*, 2015a; Heckeroth & Tenter, 1998; Ortega-Mora, 2007). *Sarcocystis* are known to mainly cause lesions and disease in the intermediate host, and the severity of sarcocystosis is influenced by the species present, the parasite infection load, localization of lesions, and the stress levels of the infected animals (e.g. pregnancy and in case of lack of food) (Dubey *et al.*, 2015a; Prakas & Butkauskas, 2012).

No specific clinical symptoms have been characterized, as sarcocystosis is usually mild in animals living under natural conditions. However, clinical signs may include weakness of the animals, weight loss, anaemia, fever and diarrhoea (Dubey *et al.*, 2015a). In cases of severe sarcocystosis, sheep may suffer from hepatitis, encephalitis,

or encephalomyelitis (Dubey *et al.*, 2015a; Fayer, 2004; Prakas & Butkauskas, 2012). In sheep, S. *tenella* is the most pathogenic species and may cause clinical signs, such as abortions, premature birth, neural signs, myositis, and may lead to death in severely infected animals (Dehaghi *et al.*, 2013; Dubey *et al.*, 2015a; Schock *et al.*, 2012). Moreover, the study by Schock *et al.*, (2012), reported a severe respiratory disease due to acute infection of *S. tenella* in 150 five to seven months old sheep from a farm in the UK. On the other hand, *S. ariticanis* and *S. gigantea* are only mildly pathogenic, and infections are usually chronic or subclinical in sheep (Bahari *et al.*, 2014; Dubey *et al.*, 2015a).

Prevalence rates of *Sarcocystis* infections in sheep worldwide can vary between different countries, and can range from an undetectable levels to 100%, showing that the genus *Sarcocystis* is widespread in varing countries (Dubey *et al.*, 2015a). In the study by Bahari *et al.*, (2014), 100% (40/40) of muscle samples were shown to be positive for *Sarcocystis* spp. of which microscopic cysts were identified as *S. tenella,* and macroscopic cysts belonged to *S. gigantean*. In the study in Saudi Arabia, *Sarcocystis* spp. were identified in 85.8% (2790/3250) of domestic sheep for which microscopic cysts were identified as *S. arieticanis*, and macroscopic cysts were identified as *S. gigantean* (Al Quraishy *et al.*, 2014). A lower prevalence of *Sarcocystis* spp., was seen by Martinez-Navalon *et al*., (2012) in a study in Spain, who observed macroscopic cysts in 12.4 % (712/5720) of sheep in 60% of sheep flocks, and identified cysts as *S. medusiformis* and *S. gigantean*. Generally, *S. gigantea* and *S. medusiformis* has been reported less frequently compared to *S. tenella* and *S. arieticanis*. It has been suggested that *Sarcocystis* species transmitted by felids were less prevalent than those transmitted by canids, as cats were reported to be a poor producer of sporocysts and they required longer incubation time to become infected (Dubey *et al.*, 2015a; Gual *et al.*, 2017).

1.4.3. Water Buffalo (*Bubalus bubalis*)

Other than cattle's, water buffalo has been found to be a natural intermediate host for *N. caninum* and infections have shown significant impact on economic production in several countries (Chryssafidis *et al.*, 2011; Konrad *et al.*, 2012; Reichel *et al.*, 2015). Water buffalos are very important as they are used for their milk, dairy products and

meat, and infection can therefore cause significant losses (Reichel *et al.*, 2015). Experimental infections have shown that vertical transmission in pregnant water buffalo can occur, and may cause abortions and foetal death (Auriemma *et al.*, 2014; Konrad *et al.*, 2012; Rodrigues *et al.*, 2005). Despite the fact that *Neospora* infections and abortions in water buffaloes are rare, lesions in the placenta, brain, heart and other organs are similar to those seen in bovine neosporosis (Auriemma *et al.*, 2014; Canton *et al.*, 2014; Dubey & Schares, 2006; Guarino *et al.*, 2000; Nasir *et al.*, 2011).

Various studies have reported different prevalence incidence of *Neospora* infections in water buffalo in different farms, ranging from a low percentages of 1.5% (3/200) in a study in southern Vietnam (Huong *et al.*, 1998), 3.8% (4/105) in a study in the Philippines (Konnai *et al.*, 2008), 9.1% (57/628) in a study in Thailand (Kengradomkij *et al.*, 2015), to higher prevalence of 88% (169/192) in a study in Brazil (Chryssafidis *et al.*, 2011), 88.3% (424/480) in a study in Australia (Neverauskas *et al.*, 2015), and 43% (584/1350) in a larger study in Argentina (Campero *et al.*, 2007). Many reports have shown that when cattle and buffalo inhabit the same area, water buffalo have a higher seroprevalence; approximately 1.5 times higher than cattle (Moore *et al.*, 2014).

Water buffalo are not only known to be infected with *N. caninum*, but infections of *T. gondii* have also been reported (Da Silva *et al.*, 2017). However, like cattle, water buffaloes are rarely affected by clinical toxoplasmosis and seem to have a natural resistance to clinical toxoplasmosis (Auriemma *et al.*, 2014; Esch, 2010). Many reports have detected DNA and antibodies against *T. gondii* in water buffaloes (Auriemma *et al.*, 2014; Da Silva *et al.*, 2017; Esch, 2010). The risk of *Toxoplasma* infections can be high particularly in countries that lack modern farming techniques. Studies have reported a seroprevalence of *T. gondii* in water buffalos ranging from undetectable to 100% in various parts of the world (e.g. including Brazil, Vietnam, Egypt and Mexico) (Alvarado-Esquivel *et al.*, 2014; Dubey *et al.*, 1998; Fujii *et al.*, 2001; Gondim *et al.*, 1999; Huong *et al.*, 1998). Antibodies to *Toxoplasma* were found for 35.5% (1715/4796) in water buffaloes in a study in Brazil (Santos *et al.*, 2013), 25.4% (127/500) of samples detected positive antibodies for *T. gondii* in a study in Argentina (Konrad *et al.*, 2013), and 14.3% (53/300) of buffalos showed *T. gondii* antibodies in a study in Iran.

Mixed infections of *T. gondii* and *N. caninum* in water buffaloes have also been reported and as they can more frequently occur it is essential to establish which species was the cause of the disease. For example, in the study by Da Silva *et al.*, (2017), water buffaloes had seroprevalence rates of 44% for *Neospora* and 39% for *Toxoplasma*. Approximately, 14.8% of water buffalo samples have been reported to show evidence of infection with both *Neospora* and *Toxoplasma* in a study by Da Silva *et al.*, (2017) in ten provinces of Brazil.

Sarcocystis infections in water buffalo are as common as in cattle. Four main *Sarcocystis* spp. have been identified, *S. fusiformis* and *S. buffalonis* (for which cats are the definitive host), *S. levinei* (for which dogs have been considered the definitive host) and *S. dubeyi* (for which the definitive host has not yet been identified) (Dissanaike & Kan, 1978; Dubey *et al.*, 2015a; Dubey *et al.*, 2014; Dubey *et al.*, 2015b; Huong *et al.*, 1997). A wide range of *Sarcocystis* spp. has been reported in various countries, and multiple infections of more than one *Sarcocystis* species have been observed by microscopy, histology and PCR (Dubey *et al.*, 2015a). In the study by El-Seify *et al.*, (2014) in Egypt, 371 water buffalo samples were examined, and 68.2% (n= 253) and 13.2% (n= 49) have shown evidence of *S. fusiformis* and *S. buffalonis* infections, respectively. Around, 83.8% (n= 311) of samples were infected with both species (El-Seify *et al.*, 2014). In the study by Oryan *et al.*, (2010) in Iran, 83% of muscle samples from buffaloes were infected with *S. buffalonis, S. levinei* and *S. dubeyi*. In another study from Selangor in Malaysia, 12/18 (66.7%) samples of water buffalo slaughtered in an abattoir were shown positive for *S. fusiformis* and *S. cruzi* (Latif *et al.*, 2013).

More recently another study, presented a new species of *Sarcocystis* '*S. dehongensis',* which was reported in 6.7% (51/756) of water buffalo by microscopy and DNA sequencing from 6 farmers markets in China (Chen *et al.*, 2017). Even though a high range of prevalence of *Sarcocystis* have been reported in water buffalo, none of the *Sarcocystis* species are considered pathogenic as no clinical disease of acute sarcocystosis has been reported (Dubey *et al.*, 2015a). Some *Sarcocystis* species found in cattle, including *S. hominis* and *S. cruzi*, might also infect water buffalo (Jehle *et al.*, 2009; Yang *et al.*, 2001a; Yang *et al.*, 2001b). This may be of interest as humans

can act as aberrant intermediate hosts for those species but their zoonotic potential in water buffaloes is unclear (Dubey *et al.*, 2015a; Dubey *et al.*, 2014).

1.4.4. Goats (*Capra aegagrus hircus*)

Goats, like sheep can be infected with *Toxoplasma*. They show similar clinical signs, and they may suffer perinatal death and abortions (Munday & Mason, 1979). Dubey (1982), described the recrudesce of a chronic infection and transplacental transmission of the parasite to the offspring in goats. In the study six does that were inoculated with the *T. gondii* strain GT-1 and gave birth to kids transplacentally infected with *Toxoplasma* and the following breeding season 2 does gave birth to kids infected with *T. gondii*, which were born dead or died shortly after birth (Dubey, 1982). Similar prevalence of *T. gondii* infection in sheep were also observed in goats ranging from undetectable to 100%. For example, in a study in Rumania 52.8% (388/735) of goats were seropositive for *T. gondii* antibodies (Iovu *et al.*, 2012). In other studies, the seroprevalence ranged from 30.2 to 100% in goats from various farms in Poland (Czopowicz *et al.*, 2011; Panadero *et al.*, 2010). In the study by Diakoua *et al.*, (2013), specific *T. gondii* antibodies were detected in 61.3% (230/375) of goats from different farms in Greece. The study by Almeria *et al.*, (2018) reported a slightly lower seroprevalence of only 5.6% (5/89) of goats from 29 farms in the Mediterranean ecosystem.

Goats have also been shown to be infected with *N. caninum*, causing clinical disease (Dubey, 2003; Porto *et al.*, 2017). However, the rates of *N. caninum* infections in goats have not been documented as well as in the other ruminants (cattle and sheep), and hence the pathogenesis remains largely unknown (Iovu *et al.*, 2012; Porto *et al.*, 2017). In the study by Porto *et al.*, (2016), goats were experimentally infected with the Nc-Spain7 isolate that was previously shown to be virulent in cattle and sheep, showed that caprine neosporosis in goats is possible. Results have also shown that the clinical outcomes of *Neospora* infections in goats depends on the stage of gestation, which is similar to cattle and sheep infected with *N. caninum* (Arranz-Solis *et al.*, 2015; Caspe *et al.*, 2012; Porto *et al.*, 2016). Infections in the initial term of gestation results in abortions, and foetal death, due to foetal tissue damage and parasite proliferation. Whereas infections during late gestation resulted in premature births, weak kids or

clinically infected but normal kids (Porto *et al.*, 2016; Porto *et al.*, 2017). Natural infections with *Neospora* in goats have been reported in several studies worldwide and seroprevalence range from undetectable to 60% in various studies (Czopowicz *et al.*, 2011; Eleni *et al.*, 2004; Moore *et al.*, 2007; Porto *et al.*, 2017). In the study by Iovu *et al.*, (2012) in Rumania, 12 out of 512 (2.3%) goats were positive for *N. caninum* antibodies. In a study in Greece, 6.9% of goats showed positive antibody titres for *Neospora* (Diakoua *et al.*, 2013). In a study in Poland, a seroprevalence of 9% was detected for *Neospora* infected goats (Czopowicz *et al.*, 2011).

In domestic goats, three main species of *Sarcocystis* have been identified based on the morphological structure: *S. capracanis* (known to cause macroscopic cysts), *S. hiricanis* and *S. moulei* (known to cause microscopic cysts) (Amairia *et al.*, 2018; Dubey *et al.*, 2015b). *Sarcocystis capracanis* and *S. hiricanis* are known to have dogs as their definitive hosts, whereas *S. moulei* is known to have cats as its definitive host (Dubey *et al.*, 2015a). Of the three species, *S. capracanis* was identified as the most pathogenic, and it has been shown to cause fever, weakness, anorexia, weight loss, abortions and death of goats, whereas no clinical signs were reported from the other two species (Dubey *et al.*, 2015a; Prakas & Butkauskas, 2012). Just like *Sarcocystis* infections in sheep, the severity of infections in goats depends on the number of sporocysts ingested.

High prevalence of *Sarcocystis* microscopic and macroscopic cysts have been recorded in goats (Dubey *et al.*, 2015a). In a study in Riyadh, in Saudi Arabia, 77% of goats slaughtered for meat consumption were shown to have natural infections with *Sarcocystis* species (Al-Hoot *et al.*, 2005) and in the study by Amairia *et al.*, (2018) infections with *Sarcocystis* were reported to be 50.4% from 121 slaughtered goats from a regional slaughterhouse in north-west Tunisia. Moreover, a study in south-western China, 77.3% (174/225) of goats from three abattoir were shown to be positive for *Sarcocystis* of which, 74.6% were identified as *S. capracanis*, and 33.3% were identified as *S. hiricanis* (Hu *et al.*, 2016). A study in Brazil examined tissue for *Sarcocystis* spp, by light and electron microscopy, macroscopic evaluation and molecular tests and detected microscopic cysts of *Sarcocystis* spp. in 91.6 % (110/120) of goat sample tested (Bittencourt *et al.*, 2016). The prevalence of *S. moulei* seems to be relatively low in comparison to the other *Sarcocystis* species identified in goats.

This was seen a study, where only 11.7% (46/393) of macroscopic cysts were identified as *S. moulei* in mountain goats from northern and central Jordan (Abo-Shehada, 1996).

1.5. Disease in Wildlife

Although, *Neospora* is a primary disease in cattle and dogs and *Toxoplasma* is a primary disease in sheep and cats, our understanding of their role in wildlife species is greatly increasing (Almeria, 2013; Gondim, 2006). The protozoan parasites *Neospora*, *Toxoplasma* and *Sarcocystis* spp. are known to infect a range of various wildlife species including wild carnivores / omnivores, such as mustelids, canids and felids and herbivores species, such as cervidae, wild ruminants and marine mammals (Almeria, 2013; Donahoe *et al.*, 2015; Dubey, 2003; Dubey, 2010). A list of wildlife species infected with *T. gondii*, *N. caninum* and *Sarcocystis* spp., can be viewed in Dubey *et al.* (2015a), Dubey (2010), Donahoe *et al.* (2015) and Dubey *et al.* (2007). In carnivore species, transmission mainly results from the ingestion of tissue cysts from infected intermediate hosts, and by ingestion of sporulated oocysts from contaminated food or water sources excreted from the definitive host. Carnivores shed oocysts in the environment, suggesting their potential role as definitive hosts (Almeria, 2013; Donahoe *et al.*, 2015; Dubey & Lindsay, 2006). Various studies have shown that multiple protozoan species in the same hosts are possible. In the study by Bartley *et al.*, (2013) and Burrells *et al.*, (2013), samples from various tissues (brain, tongue, neck muscle, heart, liver, leg muscle, lung, spleen and spinal cord) from the same carnivore species were examined for protozoal species. *Neospora* DNA was identified in 18.6% (13/70) of polecats, 10.9% (7/64) of badgers 10.1% (10/99) of ferrets, 4.8% (4/83) of foxes and 4.6% (3/65) of mink (Bartley *et al.*, 2013). In the same samples, *Toxoplasma* DNA was detected in 23.2% (23/99) of ferrets, 6% (5/83) of foxes, 31.4% (22/70) of polecats, 29.2% (19/65) of minks, 25.0% (16/64) of badgers and 44.4% (4/9) of stoats (Burrells *et al.*, 2013).

The transmission of protozoan parasites in herbivore species is mainly vertical transmission of tachyzoites from mother to offspring, and horizontal transmission via ingestion of sporulated oocysts, suggesting that herbivores mainly play a role as

intermediate hosts for these protozoan parasites (Donahoe *et al.*, 2015). Transmission and clinical disease in ruminants is well documented. However, infections and clinical disease in many wild species remain largely unknown. A high prevalence of infections and antibodies from protozoan parasites in domestic animals is relatively common and have been reported all over the world, yet there are relatively few reports of clinical disease of toxoplasmosis, neosporosis and sarcocystosis in wildlife species (Dubey, 2010; Dubey & Schares, 2011).

Clinical manifestations in wildlife depend on a wide range of factors, such as host immune status, age, parasite species, as well as dose, life stage and route of infection (Donahoe *et al.*, 2015; Dubey, 2010). Moreover, in an area where the seroprevalence to protozoan parasites is high, clinical signs are less frequently seen as antibodies against the parasite may already be high and reoccurring infection may only trigger a secondary immune response. This can subsequently lead to a higher resistance against protozoan parasites. On the other hand, in wildlife animal species which live in an environment that is relatively free of definitive hosts have a relatively low parasite burden (i.e. *T. gondii* and *N. caninum*). However, once these animals are infected, clinical signs of the disease are more likely to occur as the immune response generally takes longer to develop antibodies as it is a primary immune response.

Results in a small study observed by Ferroglio *et al.*, (2014) reported a higher infection rate in carnivores, red fox 20.21% (19/ 94) and wild boars 16.19% (17/105) compared to ruminant species, 2.48% (3/121) in roe deer and undetectable levels in red deer (0/13). A significant higher prevalence of protozoan infections has been found in carnivores species compared to herbivores and omnivores, potentially due to a higher probability of a carnivore consuming tissue from infected animals than a herbivore ingesting oocysts from the contaminated environment (Ferroglio *et al.*, 2014). This was also demonstrated in the study by Smith & Frenkel (1995), who reported higher antibodies against *Toxoplasma* in 66% (38/58) of carnivores compared to 15% (14/94) in omnivores and 11% (13/117) in herbivores. Moreover, another study by Witkowski *et al.,* (2015), who inspected meat juice selected from game animals reported similar tends and showed higher detection of antibodies against *T. gondii* in wild boars 37.6% (138/367) compared to 30.4% (28/92) in roe deer and 24.15% (133/552) in red deer.

These studies showed that *T. gondii* present in wildlife may be a potential source of infection for humans.

Studies of wildlife animals infected with *Sarcocystis* species are less frequently reported than in ruminants, as ruminant species are most commonly monitored and infections are usually detected at post-mortem meat inspections (Dubey *et al.*, 2015a). Accurate assessment of the prevalence in each host population is mainly developed in domestic animals, whilst in wildlife species detection methods for protozoan parasites have not been validated, and hence the prevalence of protozoan infections in wildlife are likely to be underestimated. Having accurate measurements of prevalence and incidence of protozoal infections in each host population could help to support prevention and the control of multi-host pathogens.

1.6. Disease in Humans

Like ruminants, humans can become infected by either the ingestion of sporulated oocysts in contaminated food, water or cat faeces, by vertical transmission of tachyzoites from mother to offspring, but mainly through the consumption of tissue cysts from raw or undercooked meat from infected animals (Dubey & Schares, 2011; McCann *et al.*, 2008; Sibley *et al.*, 2009).

Toxoplasmosis in humans was first recognized in the 1930s, and it was shown that the *T. gondii* isolates found, belonged to the same species than those previously described in animals (Sabin, 1939). Since then, *Toxoplasma* has been estimated to have infected up to one-third of the world population (Dubey, 2010; Tenter *et al.*, 2000). According to Scallan *et al.* (2011), *T. gondii* infections in humans are responsible for approximately 24% of all deaths attributed to foodborne pathogens, and have accounted for 327 deaths per annum in the USA from 2000 to 2008. Moreover, an estimated 10,964 loss in quality-adjusted life years, a $2,973 million cost due to illness and 4,428 hospitalizations per annum were observed (Scallan *et al.*, 2011). In healthy individuals (immunocompetent), infections with *T. gondii* are largely subclinical or asymptomatic, and in the majority of cases, the route or time of transmission is mainly undetermined in the majority of cases (Hill & Dubey, 2002). Clinical signs are less severe than in immunocompromised individuals, and infected humans have shown flu-

like symptoms, such as fever, swollen lymph nodes, muscle aches and sore throats (Shaapan, 2016). A higher risk of toxoplasmosis can be seen in specific groups: in congenitally infected children, immunocompromised individual (those with AIDS, cancer or organ transplant patients) and in pregnant women (Cong *et al.*, 2015; Guo *et al.*, 2015; Saadatnia & Golkar, 2012).

Congenital toxoplasmosis is mainly the results of vertical transmission of *T. gondii* (tachyzoites) during pregnancy from mother to child via the placenta. The severity and risk of infections from the disease depend on the stage at which the mother becomes infected or the immune status of the patient (McAuley, 2014; Tenter *et al.*, 2000). Even though primary infections in pregnant women are mostly asymptomatic, the parasite might cross the placenta, infecting the foetus and can damage the foetus with consequences, such as retinochoroiditis, hydrocephaly, mental retardation, seizures, abortions and even foetal death (Dubey, 2010; Saadatnia & Golkar, 2012; Tenter *et al.*, 2000). It has been estimated that the prevalence of prenatal infections with *Toxoplasma* can vary from 1 to 100 per 10,000 births in different countries (Dubey, 2010; Hayde & Pollak, 2000; Roberts *et al.*, 1994; Tenter *et al.*, 2000). In a study in France, congenital toxoplasmosis has been estimated to occur in 3.3 per 10,000 births, and in a study in Brazil, it was estimated to be 1 in 3000 births (Neto *et al.*, 2000; Villena *et al.*, 2010). Moreover, higher foetal transmission rates have been observed in the second to third trimester ranging between 33 - 47% to 60 - 81% compared to 6% in the first trimester in a study in France (Dunn *et al.*, 1999).

In immunocompromised patients, with immunodeficiency conditions, such as AIDS, cancer, and organ transplant patients, the most common cause of infections is due to the reactivation of a current infection (Dubey, 2010; Innes, 2010; McAuley, 2014; Wang *et al.*, 2017a). This reactivation results from the conversion of the bradyzoites stages to the active and rapidly replicating tachyzoite stage, resulting in fatal tissue injuries. Clinical signs and symptoms tend to be more severe, ranging from seizures, cerebral haemorrhage, toxoplasmic encephalitis or pneumonitis, myocarditis, and chorioretinitis, which are often fatal, producing organ failures and deaths (Dubey, 2010; Guo *et al.*, 2015; Kim & Weiss, 2004; Petersen *et al.*, 2010; Saadatnia & Golkar, 2012). Screening of HIV-infected (immunocompromised) women has been suggested, in order to minimize the deleterious effect of the *T. gondii* infections (Zeleke &

Melsew, 2017). The study by Wang *et al.*, (2017b) involved 312 studies of 25,989 HIV-infected patients from 34 countries showed a prevalence of 35.8% (n= 7326) for *T. gondii*. Another study in Iran, involving the pooled results of eight publications of 1,032 HIV / AIDS-immunocompromised individuals showed an overall *Toxoplasma* prevalence of 50.1% in HIV individuals, and transplant patients showed a *Toxoplasma* prevalence of 55.1% of 702 cases (Ahmadpour *et al.*, 2014; Yohanes *et al.*, 2014).

Ocular toxoplasmosis is known to be acquired mainly from prenatal infection with *T. gondii* during pregnancy and is due to the reactivation of congenital infections at a later stage (Bosch-Driessen *et al.*, 2002; Butler *et al.*, 2013). Various clinical symptoms have been reported, including visual impairment, such as blurred vision, red eyes, scotoma, pain, retinochoroidal scars and blindness (Bosch-Driessen *et al.*, 2002; Butler *et al.*, 2013). The prevalence of ocular toxoplasmosis has been estimated to be between 4% and 18% of all uveitis patients in different studies from different countries (Butler *et al.*, 2013; Jakob *et al.*, 2009; Schluter *et al.*, 2014). In 2001 in the USA, approximately 1.26 million patients were estimated to have acquired ocular toxoplasmosis, in a population of 280 million (Holland, 2003). Further prevalence data of *T. gondii* infection in humans have been reviewed by Butler *et al.* (2013), Schluter *et al.* (2014), Tenter *et al.* (2000) and Webster (2010).

The prevalence of *Toxoplasma* infections varies depending on countries, geographical areas, and ethnic groups (Dubey, 2009; Dubey, 2010; Saadatnia & Golkar, 2012; Tenter *et al.*, 2000). Epidemiological evidence showed that a higher prevalence of *T. gondii* is present in people from countries or regions where the consumptions of raw or undercooked meat are common practice (Dubey, 2009; Dubey, 2010; Dubey & Beattie, 1988). A study in Australia demonstrated that the occurrence of a congenital infection outbreak was due to the consumptions of undercooked lamb and kangaroo meat (Robson, 1995). In a study in the UK, *T. gondii* DNA was detected in 38% (27/71) of meat samples of which 6/9 lamb samples, 1/4 beef samples and 19/58 pork samples all obtained from a commercial source, were positive (Aspinall *et al.*, 2002). Another study in the Netherlands reported that 7.1% of meat-borne infections were infected in humans were cause by *T. gondii*, of which 67.6% of meat originated from beef, 11.2% from pork and 14% from lamb, (Opsteegh *et al.*, 2011a). A high percentage of the world's population is estimated to be infected with *Toxoplasma*,

however, the prevalence of *T. gondii* in immunocompetent people is underestimated. Detection of *T. gondii* in immunocompetent people is rarely recorded, as clinical signs are seldom observed and thus infections is only detectable if mild clinical signs are detected (Dalimi & Abdoli, 2012; Saadatnia & Golkar, 2012). In the study by Sucilathangam *et al.*, (2012), antibodies against *T. gondii* were detected in 10.9% (n= 19) of 175 immunocompetent pregnant patients, and in 8.9% (12/135) of pregnant women compared to 15.4% (27/175) in immunodeficient patients.

Initially, there was a concern that *Neospora* might be another zoonotic parasite, because of its close phylogenetic relationship to *Toxoplasma*, its similar life cycle and wide range of intermediate hosts (McCann *et al.*, 2008). Humans were thought to become infected by accidental exposure to oocysts in faeces from infected dogs or through the consumption of undercooked meat containing tissue cysts (Dubey & Schares, 2011; McCann *et al.*, 2008). In the study by Petersen *et al.*, (1999), 76 women who had undergone abortions were examined for *N. caninum* antibodies, but no evidence of seroconversion was detected by ELISA, immunofluorescence assay, or western blot. Similar results were shown in the study by McCann *et al.*, (2008) in England, who tested 518 serum samples from high-risk farm workers and 3,232 serum samples from the general population, and found no evidence of exposure to *N. caninum* by ELISA and immunofluorescence assay.

On the other hand, a larger study by Tranas *et al.*, (1999) in the USA showed that of blood donor sera samples 1029 samples 6.7% (n= 69) had positive *Neospora* titres by indirect fluorescent antibody testing. A smaller study by Nam *et al.*, (1998) in Korea, demonstrated seropositive rates of *Neospora* in 6.7% (12/172) of human sera samples. Evidence of *Neospora* infections in humans was demonstrated in various studies by Lobato *et al.*, (2006) and Dubey *et al.*, (2007), who showed detection of immunoglobulin G antibodies to *Neospora* in 38% (23/60) of HIV-infected patients, 18% (9/50) of patients with neurological disorders, 5% (5/91) in newborns and 6% (3/54) in healthy subjects, respectively. Even though antibodies to *Neospora* have been reported, there has been no actual evidence of the parasites in human tissues and hence the disease itself (Dubey *et al.*, 2007; Lobato *et al.*, 2006). Neosporosis is not considered a zoonotic disease, and although only limited evidence of *N. caninum* in

humans has been reported so far, potential infections of *N. caninum* in humans may occur, due to the biological similarities between *Neospora* and *Toxoplasma*.

Infections of s*arcocystis* are normally discovered during post-mortem investigations of carcasses, slaughtered for human consumptions. Infections missed during these inspections of livestock can become a public health risk (Fayer, 2004; Fayer *et al.*, 2015; Poulsen & Stensvold, 2014). Humans have been known to be infected with two main species of *Sarcocystis*, namely *S. hominis* (found in cattle) and *S. suihominis* (found in pigs) (Dubey *et al.*, 2015a; Fayer *et al.*, 2015). Humans can be definitive hosts for these *Sarcocystis* species, and the parasite can cause human intestinal Sarcocystosis via the ingestions of meat containing mature sarcocysts (Fayer *et al.*, 2015; Poulsen & Stensvold, 2014). *Sarcocystis hominis* is known to be only mildly pathogenic for humans, whereas *S. suihominis* is more pathogenic (Dubey *et al.*, 2015a; Heydorn, 1977; Piekaski *et al.*, 1978).

Clinical symptoms caused by *S. suihominis* include nausea, vomiting, stomachache, diarrhoea, dyspnoea and chronic or acute enteritis (Dubey *et al.*, 2015a; Heydorn, 1977; Piekaski *et al.*, 1978; Sabin & Olitsky, 1937). A recent study in Cambodia showed that 10% of participants (n= 1081) were infected with *Sarcocystis* spp. (Khieu *et al.*, 2017). This study also showed clinical symptoms for these patients with 73.1% reported abdominal pain and discomfort, 63% reported diarrhoea, 49.1% reported muscle pain and 48.1% reported itching skin (Khieu *et al.*, 2017). Most human infections with *Sarcocystis* spp., however, appear mild or asymptomatic and symptoms vary depending on the species and numbers of oocysts / tissue cysts ingested (Dubey *et al.*, 2015a; Fayer *et al.*, 2015). Any meat or tissues of domestic animals, wild mammals, birds or reptiles that are used for human consumption and contain sarcocysts are capable of infecting humans, and can cause accidental infections (Dubey *et al.*, 2015a; Fayer *et al.*, 2015). Humans may also act as intermediate hosts by ingestions of sporocysts from contaminated food, water or the environment that would cause muscular sarcocystosis (Beaver *et al.*, 1979; Fayer *et al.*, 2015; Poulsen & Stensvold, 2014). Human intestinal and muscular sarcocystosis has been identified worldwide. The study by Pena *et al.*, (2001) in Brazil found *Sarcocystis* species in 100% (50/50) of bovine samples. It was shown that 94%, 70% and 92% of samples were identified as *S. hominis*, *S. hirsuta* and *S. cruzi*, respectively, of which *S. hominis*

could be transmitted to humans (Pena *et al.*, 2001). In a study in part of Guangxi Provine in China showed that 27 of 501 people examined were infected with *S. suihominis* (Tan *et al.*, 2005), and the study in Thailand showed that 23.2% of 362 Thai labourers were suspected to be infected with *S. suihominis* (Wilairatana et al., 1996). Another study in Laos, Giboda et al., (1991), showed 23.2% of 1,008 labourers were infected with *S. hominis*, and a study in Malaysia showed detection of *Sarcocystis* antibodies in 19.7% of people (n= 243) (Thomas & Dissanaike, 1978). Other prevalence studies of intestinal and muscular sarcocystosis are reviewed by Dubey *et al.* (2015a) and Fayer *et al.* (2015).

1.7. Diagnostics of disease and detection of *Neospora*, *Toxoplasma* and *Sarcocystis*

In the past, protozoan species were identified solely using morphological analysis as the gold standard for the identification and diagnosis, and no molecular and genetic analysis were performed (Mangal *et al.*, 2016). Identification of species employs the structural characteristics that vary between the cysts (Dubey *et al.*, 1989b; Hamidinejat *et al.*, 2015). Different sizes amongst *Neospora*, *Toxoplasma* and *Sarcocystis* spp. parasites can frequently be seen, yet differentiation between these protozoans is difficult based only on size. For *Neospora*, the size of tachyzoites can vary from 6 x 2 μm up to 5 x 8 μm, and tissue cysts are often round and oval in shape at around 100 μm of which the wall can either be thick walled (4 μm) or thin walled (0.3 - 1 μm) (Dubey & Lindsay, 2006). The enclosed bradyzoites can be up to 8 x 2 μm and oocysts can measure up to 12 μm in diameter (Al-Qassab *et al.*, 2010; Dubey *et al.*, 2006; Dubey & Lindsay, 2006). *Toxoplasma* parasite stages are similar in size with tachyzoites being up to 2 x 6 μm; tissue cysts vary from 5 to 70 μm with enclosed bradyzoites of 0.7 to 1.5 μm and oocysts up to 10 x 12 μm (Dubey & Lindsay, 2006).

Sarcocystis are easier to distinguish from *Neospora* and *Toxoplasma* as they vary in shape and size, depending on the *Sarcocystis* spp. (Dubey *et al.*, 2015a). Some *Sarcocystis* remain microscopic (such as *S. cruzi*) varying from very long and narrow to short and wide, whereas others may become macroscopic (*S. gigantea*). They are nearly always found in the skeletal muscles appearing like rice grains, fusiform or

globular (Dubey *et al.*, 2015a). The size of the cyst and the thickness of the cyst wall can vary among species from a few micrometres to a few millimetres as the host matures (Dubey *et al.*, 2015a). Bradyzoites are often banana- shaped and vary in size, and the size has been reported to be around 7 to 9 μm (Dubey *et al.*, 2015a). Sporozoites and oocysts are slightly bigger, ranging from around 10 to 19 μm by 8 x 10 μm, and 15 x 19 μm long by 15 x 10 μm wide, respectively (Dubey *et al.*, 2015a). Sarcocysts have been shown to be differentiated by their wall, either thin walled (< 1 μm) or thick walled (> 1 μm) and can vary from being very simple to highly complex structures (Dubey *et al.*, 2015a). Moreover, the size of *Sarcocystis* is known to change depending on the infection period and host species (i.e. larger in large animals) (Dubey *et al.*, 2015a).

Using microscopic examination may be sufficient in differentiating the *Sarcocystis* genus from *Neospora* and *Toxoplasma*; however, species identification of *Sarcocystis* spp. may prove difficult as the wall structure is subject to change depending on the age of the sarcocysts and thus has 'limited applicability as a morphological criterion' (Fayer, 2004). Moreover, microscopic detection of very closely related *Sarcocystis* species may still be challenging as expert knowledge is required. Additionally, identification and diagnosis between these closely related parasites might prove more difficult to achieve, especially if mixed infections are present in the same host. Not only do the closely related protozoan *T. gondii, N. caninum* and *Sarcocystis* spp. have similar life cycles, they show similarity in morphology and ultrastructure of the infectious stages (tachyzoites, bradyzoites and sporozoites) (Hamidinejat *et al.*, 2015; Lindsay *et al.*, 1999; Speer *et al.*, 1998).

Pathology is an important tool in detecting morphological inflammatory lesions associated with protozoan parasites in tissues (e.g. brain, heart, liver, placenta, muscles tissue) (Dubey & Schares, 2006). In the study by Wouda *et al.*, (1997), eighty bovine foetuses with confirmed *Neospora* infections in the brain, heart and liver, were compared in respect of histopathological lesions and the distribution of parasites. Lesions were identified by histopathology in 91% (73/80) of sample studied. However, in the other 7 cases, lesions were only observed in two of the three tissues (Wouda *et al.*, 1997). Most histological lesions can be found in several organs, of which the brain and placenta are the main ones infected (Wouda *et al.*, 1997).

However, diagnosis of protozoan infections using histopathology can be difficult as tachyzoites and bradyzoites are not always associated with inflammatory lesions in the samples tested. In *Neospora*, the number of parasites found in bovine tissue generally tends to be low (Dubey *et al.*, 2015a; Dubey & Schares, 2006). Detection of tachyzoites using routine histology Haematoxylin and Eosin (H&E) may prove difficult even when the tissue is well preserved (Dubey & Schares, 2006). Even if apicomplexan-like protozoa are found in the brain of an aborted bovine foetus, it does not necessarily indicate that *N. caninum* was the cause of abortion as other aetiological agents could cause similar lesions.

Using histological analysis may be sufficient to identify the cause of abortion when characteristic lesions are present, however differentiation between the protozoal parasites may require a more aetiologic diagnostic techniques such as immunohistochemistry (IHC) or polymerase chain reaction (PCR) (Dubey, 1986; Dubey & Lindsay, 1996; Dubey & Schares, 2006; Gonzalez *et al.*, 1999). When demonstating the presecne of the parasite, IHC labelling is a more reliable method of diagnosis than conventional H&E staining and should be used to demonstrate the presence of the protozoan parasite (Boger & Hattel, 2003). Silva *et al.* (2013) reported that using H&E staining of naturally infected *T. gondii* tissues did not reveal the presence of parasite, yet tissues were positive using IHC labelling. This is due to the difficulty to detect low parasite numbers using H&E stain to (Dubey *et al.*, 2006). Antibodies for parasite-specific antigens are key to increase the specificity of diagnosis, detecting and differentiating *T. gondii, N. caninum* and *Sarcocystis*, (Borel *et al.*, 2014; Dubey & Lindsay, 2006).

1.7.1. IHC detection of protozoan parasites

IHC is a diagnostic method that localises specific antigens in histological sections by using antigen-specific antibodies for the detection of parasite-antigens, hence the identification of parasite itself. This makes IHC a more accurate method of parasite identification than conventional H&E stain (Cabral *et al.*, 2009; Dubey & Schares, 2006; Pescador *et al.*, 2007a; Taylor & Burns, 1974). Fixed tissues are an excellent source of phenotypic analysis, as it preserves the morphological structures. IHC diagnostics allow localisation and visualisation of the parasites within tissue sections

showing the distribution of the pathogen (Haines & West, 2005; Ramos-Vara *et al.*, 2008). IHC can be used either directly or indirectly. The direct method uses specific antibodies conjugated with biotin that binds to labelled avidins, which are used to develop the chromogen reaction (Schacht & Kern, 2015). Staining is amplified due to the secondary layer, increasing the sensitivity of labelling. The indirect method uses two layers of antibodies: a primary antibody that enables detection of specific parasite epitopes and a secondary that has the specificity for the antibody species of the primary being used. IHC employs polyclonal (pAb) or monoclonal (mAb) antibodies for the detection of parasites.

At first no cross-reactivity between the apicomplexan parasites *N. caninum, T. gondii* and *Sarcocystis* spp. was reported and a potentially misdiagnosis may have occured (Canada *et al.*, 2002). However, more recent comparisons have revealed false diagnoses of *T. gondii* in *N. caninum* infected animals (Van Maanen *et al.*, 2004). Various cross-reactive antigens have been detected so far. For example a *T. gondii* bradyzoites specific antigen (BAG5) that has shown cross-reactivity with the same antigen of *N. caninum* and *S. cruzi*, a rhoptry protein (42kDA) protease of *T. gondii* that was shown similar to that of *Neospora* (Ahn *et al.*, 2001), a *Neospora* dense granule protein (NTPase) that showed cross-reactivity with *T. gondii* (Asai *et al.*, 1998), as well as various surface proteins have shown a high homology between *Neospora* and *Toxoplasma* (McAllister *et al.*, 1996b; Peters *et al.*, 2001). Total parasite lysates have frequently been used for the production of antibodies. However, total parasite lysates from tachyzoites or tissue cysts are morphological similar and share homologous antigens, and antibodies produced against them are very likely to cross-react. It has been reported that polyclonal antibodies produced for *Neospora* IHC showed cross-reactivity with other cyst-forming parasites (*T. gondii* and *S. cruzi*) (McAllister *et al.*, 1996b; Peters *et al.*, 2000; Ramos-Vara *et al.*, 2008).

Using specific recombinant proteins could reduce the risk of cross-reactivity, as antibodies are produced against a single antigen. In the study by Uzeda *et al.*, (2013), various antigens (recombinant proteins) were tested in combination with monoclonal and polyclonal antibodies to detect *N. caninum* antigens and showed an increased specificity for the detection of *Neospora* and thus preventing cross-reactivity between *Neospora, Toxoplasma* and *Sarcocystis* (Van Maanen *et al.*, 2004). Various antigens

and protein targets, such as SAG and SRS genes have been previously described for the development of diagnostic tests for *Neospora* (Ellison *et al.*, 2002; Garcia-Bocanegra *et al.*, 2013; Howe *et al.*, 1998; Howe *et al.*, 2002; Jung *et al.*, 2004; Khanaliha *et al.*, 2014; Risco-Castillo *et al.*, 2011; Sa *et al.*, 2014; Sabaj *et al.*, 2010; Schares *et al.*, 2000), for *Toxoplasma* (Van *et al.*, 2007) and for *Sarcocystis* spp. (Howe *et al.*, 2005; Zhang & Howe, 2008) and those could be used to develop recombinant proteins that could be used to raise specific antibodies for *Neospora*, *Toxoplasma* and *Sarcocystis* spp. for IHC analysis.

1.7.2. Molecular detection of protozoan parasites

PCR is a method developed in the 1980s by Kary Mullis for the identification and amplification of DNA from various infectious diseases, including protozoan DNA of various animal tissue samples (Thompson & Kowalski, 2011). PCR allows a specific region of DNA to become synthesized and multiplied, thus generating a complimentary copy of the given template (Mullis *et al.*, 1986). For the detection of protozoan DNA, various samples (i.e. amniotic fluid, cerebrospinal fluid, faeces and blood) from infected cattle were examined for *N. caninum* infection using a single round PCR. However, results were poor and DNA was not recognised by a single round of PCR due to the low parasite burden in the samples (Dubey & Schares, 2006; Guy *et al.*, 2001). Single-round PCRs are available for detection of *N. caninum*, however, a more sensitive detection method is required to detect smaller quantities of DNA in the presence of excess mammalian DNA (Hughes *et al.*, 2006). This was also suggested by Duncanson *et al.* (2001) and Collantes-Fernandez *et al.* (2002), from work on *T. gondii* in host tissues, who proposed that the use of a nested PCR reaction would create a more sensitive detection of apicomplexan parasites. It was suggested by Ellis (1998), that universal primers might be needed in the first round PCR reaction, and more specific primers in the second round to enable a more sensitive and specific detection method to differentiate between protozoan parasites and host cell DNA when low parasite infections are present. Using universal primers could enable simultaneous amplification of DNA from a range of protozoan species, yet exclude host DNA and enable the detection of mixed parasitic infections. With more specific inner primers,

detection of specific protozoan species could be achieved, increasing sensitivity and specificity using a nested round of PCR.

Various target regions to establish species-specific primers have been previously described. Choosing a multi-copy gene region confers an advantage, as it has a higher analytical sensitivity compared to single copy genes (Dubey & Schares, 2006). Ribosomal RNA (rRNA) gene has thus become an important target for diagnostics, due to its repetitive characteristics of coding genes, such as 18S rRNA gene, ITS1 region and the pNc5 gene (Dubey & Schares, 2006). Many studies have used the repetitive DNA sequence of the pNc5 gene as a target for *N. caninum*. However, no corresponding genes seem to exist in the *T. gondii, S. cruzi,* or *H. hammondia* genomes (Dubey & Schares, 2006; Muller *et al.*, 2001). In the study by Gottstein *et al.*, (1998), the pNC-5 gene was used to detect *Neospora* infections in 83 aborted bovine foetuses, yet poor correlation between the PCR positive results and histologic lesions was observed potentially due to low sensitivity. The pNC-5 gene can only detect *Neospora* DNA, however it is unable to identify or differentiate if any other protozoan species (*Sarcocystis* or *Toxoplasma*) are present.

Another area the 'ITS1 region' has been used for the detection of protozoan DNA, especially for the differentiation between *Neospora* and *Toxoplasma*. In the study by Ellis (1998), 16 of 40 positive *N. caninum* cases were identified by ITS1 in foetuses with heart and brain lesions. In the study by Bartley *et al.*, (2013) and Burrells *et al.*, (2013), *T. gondii* and *N. caninum* DNA were detected in various badger tissue samples, of which some badgers showed mixed infections of both *Neospora* and *Toxoplasma.* Thus using the ITS1 region can help to differentiate between the closely related protozoan *Neospora* and *Toxoplasma.*

Various PCR methods have been published targeting the ITS1 region, allowing species-specific diagnostics of *T. gondii, N. caninum, H. heydorni* and *H. hammondi* (Dubey & Schares, 2006). However, only limited information and only a few ITS1 sequences for the *Sarcocystis* genus are available making differentiation between the *Sarcocystis* genus from *Neospora* and *Toxoplasma* considerably difficult (Stojecki *et al.*, 2012). Nevertheless, more information on the 18S rRNA gene for *Sarcocystis* spp. is becoming available, helping to differentiate *Sarcocystis* species DNA and potential

Sarcocystis infections from *Neospora* and *Toxoplasma*. The 18S gene has shown a lot of polymorphic regions and conserved regions for the genus *Sarcocystis, Toxoplasma* and *Neospora*, making the development of universal and genus-specific primers possible to enable detection of mixed protozoan infections. Yet the 18S gene has shown some limitations for very closely related species. Only minor differences have been found between *Neospora* and *Toxoplasma* in the 18S rRNA gene for the development of species-specific primers, making potential differentiation between these two challenging using only the 18S rRNA gene (Marsh *et al.*, 1995). Even though PCR is a unique tool for the identification and speciation of protozoan DNA, it is not sufficient for the diagnosis of disease on its own as it only identifies the parasite DNA, and does not show that the parasite is the cause of disease. PCR diagnostics should be used in conjunction with other techniques such as histopathology and IHC to determine which protozoan species is present, and if this protozoan was the cause of abortion.

1.8. Project aims and thesis outline

Abortion caused by protozoan parasites is a major problem for livestock operations and animal welfare worldwide, and the diagnosis of the parasites is particularly difficult. Accurate monitoring programs are required to distinguish between these parasites and confirm the presence or absence of protozoal parasite, and to estimate the levels of infection within a herd in order to adopt the most relevant disease control strategy. As such, it is necessary to have access to specific diagnostic tools in order to confirm or rule out the presence of *Toxoplasma gondii*, *Neospora caninum,* and *Sarcocystis* spp. as the cause of abortion. This decision-making process is hindered by the absence of reliable diagnostic methods. As protozoan parasites show a wide distribution in a variety of different hosts and can cause clinical disease in varied ruminant species, detection of *N. caninum*, *T. gondii* and *Sarcocystis* spp. cannot be ruled out.

The main aim of this study was to improve the diagnosis of abortion cases in ruminants infected with protozoan parasites by producing reagents, such as PCR primers and antibodies that are able to better discriminate between protozoan parasites, making a misdiagnosis less likely. There are two main goals for this study:

1) Produce genus-specific antibodies raised against recombinant proteins of *T. gondii, N. caninum* and *Sarcocystis* spp. that can be used for a specific diagnosis of pathology samples from neonatal animals of abortion cases in ruminants.

2) Produce genus-specific PCR that can detect and distinguish between *T. gondii, N. caninum* and *Sarcocystis* spp. in ruminants to confirm or rule out potential infections.

Aim 1) is achieved by the following objectives.

a) Identification of suitable target genes for the production of genus-specific recombinant proteins for *Neospora, Toxoplasma* and *Sarcocystis* spp.
b) Expression and purification of recombinant proteins for the different parasites species.
c) Raising antisera against the genus-specific recombinant antigen produced for *Neospora, Toxoplasma* and *Sarcocystis* spp.
d) Testing of antisera for their species-specific diagnostic potential in immunohistochemical analysis in experimental and natural cases from the Moredun pathology and surveillance tissue archive.

Aim 2) is achieved by the following objectives.

a) Identification of a suitable target DNA region that can be used for the development of genus-specific PCRs, which are sensitive and can be used for DNA obtained from fresh / frozen and fixed tissue samples.
b) Development and design of genus-specific primers that allow diagnosis of *Toxoplasma, Neospora* and *Sarcocystis* spp. Validation of genus-specific diagnostic PCR using fresh / frozen and fixed tissues using the Moredun pathology and surveillance tissue archive.
c) Validation of genus-specific diagnostic PCRs using fresh / frozen and fixed tissues using the Moredun pathology and surveillance tissue archive.

Chapter 2.

Chapter 2.1. Improved PCR diagnostics: genus-specific PCR assay

2.1.1 Introduction

Various studies have demonstrated that ruminants can be infected by *Neospora*, *Toxoplasma* and *Sarcocystis* spp., which are known to cause abortions in livestock (Dubey, 2010; Dubey *et al.*, 2015a; Dubey *et al.*, 2014; Dubey & Schares, 2011). However, the diagnosis of protozoan infections has proven difficult even in the presence of clinical disease, due to the limited clinical signs observed in animals infected with *Neospora*, *Toxoplasma* and *Sarcocystis* (Dubey & Schares, 2006). The finding of an apicomplexan-like protozoan in the brain from an aborted bovine foetus, does not necessarily indicate that *N. caninum* was the cause of abortion, as other Apicomplexa parasites, such as *T. gondii* or *Sarcocystis* spp. could be present and thus the cause of abortion. Various studies have demonstrated that ruminants can be infected by *Neospora*, *Toxoplasma* and *Sarcocystis* spp., which are known to cause abortions in livestock

Studies have shown that pregnant sheep are very susceptible to *N. caninum* infections, and lesions found in the foetus and placenta were similar to those seen in bovine neosporosis and ovine toxoplasmosis (Buxton *et al.*, 1997; Dubey & Lindsay, 1990; McAllister *et al.*, 1996a). *Toxoplasma* infections in cattle have also been observed, and *T. gondii* was successfully isolated from naturally infected aborted bovine foetuses (Amdouni *et al.*, 2017; Canada *et al.*, 2002). Likewise, studies have shown that ruminants, such as sheep, cattle, goat and buffalos, can be intermediate hosts to numerous *Sarcocystis* spp., of which some pathogenic species are known to cause abortions (Dubey *et al.*, 2015a; Dubey *et al.*, 2014). As such, it is necessary to have access to specific diagnostic tools in order to distinguish and identify the presence of

T. gondii, *N. caninum,* and *Sarcocystis* spp. within ruminants as a specific cause of abortions.

The diagnosis of the protozoan diseases caused by *Neospora, Toxoplasma* and *Sarcocystis* spp. is typically based on the detection of typical microscopic lesions associated with the presence of parasites in tissues by either IHC (Dubey & Hamir, 2000; Dubey *et al.*, 2007; Morales *et al.*, 2001; Silva *et al.*, 2013) or conventional PCR (Bourdin *et al.*, 2014; Sadrebazzaz *et al.*, 2007; Stojecki *et al.*, 2012). Many diagnostic laboratories use fixed and fresh tissue samples for nucleic acid extraction (Buxton *et al.*, 1998; Ellis, 1998; Sanchez *et al.*, 2009; Van Maanen *et al.*, 2004). Fixed samples are a good resource, as they allow long-term preservation of the tissue and of the infection agents, especially if DNA is not available from fresh / frozen tissues (Kokkat *et al.*, 2013; Lin *et al.*, 2009). Nevertheless, the extraction of DNA remains challenging. The fixatives, such as formaldehyde used in FFPE can lead to cross-linkage between proteins. Fixation can cause the nucleic acids to fragment, decreasing PCR yield and thus making PCR amplification of large and high molecular weight DNA fragments considerably challenging (Gilbert *et al.*, 2007; Kokkat *et al.*, 2013; Ren *et al.*, 2000). However, DNA can be successfully purified, and the amplification of fragments of less than 500 base pairs via nested PCR is possible (Lin *et al.*, 2012).

Various molecular methods have been described to detect *N. caninum, T. gondii* and *Sarcocystis* species; they target the multicopy 18S rRNA of the ribosomal RNA gene family, and the internal transcribed spacer (ITS1) region of protozoan parasites of the family Sarcocystidae from fresh tissue samples (Buxton *et al.*, 1998; Dubey & Schares, 2006; Fischer & Odening, 1998; Hurtado *et al.*, 2001; Jehle *et al.*, 2009). However, only a few methods have been able to amplify and distinguish all three protozoal parasites using fixed tissue samples using PCR, as methods for fixed tissue were shown less sensitive in comparison to fresh tissues (Buxton *et al.*, 1998; Lin *et al.*, 2000; Sanchez *et al.*, 2009; Suleman *et al.*, 2016). It is essential to develop new diagnostic tests in order to be able to distinguish between the different protozoal species.

The development of any new molecular diagnostic test involves many levels of assessment, standardizations and validation of both the diagnostic and technical use of

the test (Halling *et al.*, 2012; Mattocks *et al.*, 2010). Standardization and validation of a new molecular assay is necessary to establish if the test is fit for the intended purpose (Mattocks *et al.*, 2010). Designing a new methodology includes determining the assay reagents, such as primers and buffer solutions, but also specific factors, such as the primer design, the location of known polymorphism in the primer-binding site, the G + C content of a region of interest and fragment length (Mattocks *et al.*, 2010). It is important to establish particular measurements, test using suitable controls (positive and negative) and replication of the tests in order to determine if the PCR assay is specific to the target of interest (Mattocks *et al.*, 2010).

Once standardization of each parameter has been established, it is necessary to verify that the test performs to a suitable level of accuracy and that it can be used to confirm or rule out infections by protozoan parasites as a cause of abortion. The tests can then be used for the detection and differentiation of protozoal parasites from naturally infected individuals. In this chapter, new primers will be designed to amplify a relatively small region of DNA that allows amplification of protozoan DNA from fixed as well as fresh tissue samples in ruminants. These primers will be used for the genus and species-specific diagnosis between the protozoan parasites *N. caninum, T. gondii* and *Sarcocystis* spp. from ruminant abortion cases.

2.1.2. Aims and objectives

In this chapter genus-specific PCR primers will be developed that could be used to distinguish between closely related protozoan parasites found in ruminants. This will be achieved by:

- Identify suitable target regions for the production of species and genus-specific PCR primer for *Neospora*, *Toxoplasma* and *Sarcocystis* spp.
- Testing primer sets for specificity using *Toxoplasma, Neospora* and various *Sarcocystis* DNA.
- Testing primer sets using fresh and fixed tissue samples.
- Validate primers using archived and suspected animal cases to rule out or confirm protozoan infections.

Chapter 2.2: Materials and Methods

2.2.1 Target DNA and primer design

Sequences of the multicopy 18S coding region of the ribosomal RNA gene family and the internal transcribed spacer 1 (ITS1) regions of the ribosomal RNA gene family of *N. caninum, T. gondii* and various *Sarcocystis* spp. were collected from the following databases: the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and ToxoBD (http://toxodb.org) (**Appendix Table I**). Protozoan species were chosen based on which were most likely to occur in ruminant species (cattle, sheep, goat, and buffalo) as well as other closely related species (**Table 2.1.)**. 18S sequences of closely related species, as well as host species, *Bos taurus*, and two unrelated species were also collected (**Appendix Table I**).

Primers were designed to amplify protozoan species found in ruminants, and protozoan found in tissue samples common for *N. caninum, T. gondii* and *Sarcocystis* spp. such as brain, heart and other muscle tissues (Dubey & Lindsay, 2006). Primers were designed based on polymorphic regions and base pair differences in the sequences. The designed primers contained at least one or two unique base pair differences at the 3' prime end of each primer, to increase specificity and avoid cross-reactivity. Primers were generated to amplify a small region of less than 500 base pairs to allow amplification of DNA from fixed tissues.

Properties of each primer were checked using 'OligoEvaluator' (Sigma-Aldrich) 'http://www.oligoevaluator.com/LoginServlet' to ensure that the parameters of the primers were met. Each primer designed was kept to 17-28 bases in length. Primer melting temperatures were adjusted until each forward and reverse primer was within 5 ºC. If the differences were higher than 5ºC, the primers were adjusted at their 5' prime ends until primer sets were within 5ºC.

Table 2. 1. Representative selections of various *Sarcocystis* and related species found in ruminants according to host species and geographical distribution.

	Protozoan species	**Geographical distribution**	**Reference**
Intermediate Host	***Sarcocystis* spp.**		
Cattle (*Bos taurus*)	*S. bovifelis*	South America, Europe, (Worldwide)	(Gjerde, 2016a)
	S. hominis	South America, Europe, UK, Asia, Australia, Africa	(Dubey *et al.*, 2015)
	S. hirsuta	South America, Europe, UK, Asia, Australia, Africa	(Dubey *et al.*, 2015; Gjerde, 2016a)
	S. sinensis	Asia, Europe, America	(Gjerde *et al.*, 2016)
	S. rommeli	Asia, Europe, South America	(Gjerde, 2016b)
	S. bovini	Australia, South America	(Gjerde, 2016a)
	S. heydorni	Asia	(Hu *et al.*, 2016)
	S. cruzi	America, Europe, UK, Asia, Australia, Africa	(Schares *et al.*, 2005)
Water Buffalo (*Bubalus bubalis*)	*S. fusiformis*	Europe, Asia, South America	(Dubey *et al.*, 2015; Gjerde, 2016a; Gjerde, 2016b)
	S. levinei	Asia, Europe	(Dubey *et al.*, 2015; Gjerde, 2016a; Gjerde, 2016b)
	S. buffalonis	Asia,	(Dubey *et al.*, 2015; Gjerde *et al.*, 2016)
	S. dubeyi	Asia	(Dubey *et al.*, 2015; Gjerde, 2016b)
Sheep (*Ovis aries*)	*S. gigantea*	Australia, Europe, Asia, UK	(Dubey *et al.*, 2015)
	S. arieticanis	Australia, Europe, Asia, North America	(Dubey *et al.*, 2015)
	S. tenella	Australia, America, Europe, UK, Asia, Africa	(Dubey *et al.*, 2015)
	S. medusiformis	Australia, Asia, America, Europe	(Dubey *et al.*, 2015)
Goat (*Capra aegarus hircus*)	*S. moulei*	America, Asia,	(Dubey *et al.*, 2015)
	S. capracanis	America, Asia,	(Dubey *et al.*, 2015)
	S. hircicanis	America, Asia,	(Dubey *et al.*, 2015)
Cattle	***Hammondia* spp.**		
	H. hammondia	Worldwide	(Gjerde & Dahlgren, 2011; Schares *et al.*, 2005)
	H. heydorni	Worldwide	(Gjerde & Dahlgren, 2011; Schares *et al.*, 2005)
	H. triffittae	Europe, America	(Gjerde & Dahlgren, 2011)
Cattle / Buffalo	***N. caninum***	Worldwide	(Dubey *et al.*, 2006; Dubey & Lindsay, 2006)
Sheep / Buffalo	***T. gondii***	Worldwide	(Dubey & Lindsay, 2006)

Melting temperatures were checked using the property check from PCR Primer (Eurofins MWG Operon). Primers GC content was kept below 60% where possible, and the GC clamp was kept lower than 3, to help promote specific binding of the 3' end. It was ensured that no or only weak secondary structures were formed, and no hairpins or dimers were formed during the reaction, which could lead to poor DNA yield of the product (PREMIER Biosoft, Accellerating Research in Life Sciences). Moreover, sequence repeats and long runs were avoided where possible. Each primer was additionally checked using the primer design software Primer3Plus 'http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi' (Untergasser *et al.*, 2007). Primers were ordered and manufactured by Eurofins MWG Operon. A list of the developed primers is contained in **Table 2. 2.**

2.2.2. Multi-sequence alignments

Bioinformatic analyses, such as multiple sequence alignments, were performed to enable the comparison of homologous sequences (Sievers *et al.*, 2011). Multiple sequence alignments were performed using the BioEdit sequence alignment editor (7.1.3.0.) and Multi Sequence Alignment Tool (MUSCLE) from Clustal Omega (the European Bioinformatics Institute) http://www.ebi.ac.uk/Tools/msa/clustalo/ (Li *et al.*, 2015; Sievers *et al.*, 2011), to show the difference between the closely related Sarcocystidae species. Conserved and polymorphic regions within the 18S rRNA gene and ITS1 regions in the alignments were analysed, and base pair differences were highlighted for primer site selections.

TABLE 2. 2. PRIMER SEQUENCES USED FOR THE DETECTION OF *NEOSPORA*, *TOXOPLASMA* AND *SARCOCYSTIS* SPP. TARGETING THE 18S RRNA GENE AND THE ITS1 REGION.

Region		Primer	Name	Primer sequence	Control DNA used	
					Positive	**Negative**
18S	External	Forward	NTS-18S-F1	5′- GCC ATG CAT GTC TAA GTA TAA G -′3	*N. caninum, T. gondii, Sarcocystis* spp.	Water, Host, *Babesia, Cryptosporidium*
		Reverse	NTS-18S-R1	5′- CCT ATC ATT CCA ATC ACT AGA AAT -′3		
		Forward	NTS-18S-F2	5'- GGA TAA CCG TGG TAA TTC TAT G -'3	*N. caninum, T. gondii, Sarcocystis* spp.	Water, Host, *Babesia, Cryptosporidium*
		Reverse	NTS-18S-R2	5'- TCC CCG TTA CCC GTC AC -'3		
		Forward	NTH-18S-F2	5'- GCT AAT ACA TGC GCA CAT GC -'3	*N. caninum, T. gondii, Hammondia spp.*	Water, *Sarcocystis* spp.
		Reverse	NTH-18S-R2	5'- GTC GCA GAC CGA AGT CAA -'3		
	Internal	Forward	S-18S-G1	5'- TAT TAG ATA CAG AAC CAA CAG TG -'3	*S. gigantea*	
		Forward	S-18S-G2	5'- ATA CAT GCG CAA ATA CTA TAT TAT GT -'3	*S. hirsuta, S. buffalonis*	
		Forward	S-18S-G3	5'- TAC AGA ACC AAY ACG CTC C -'3	*S. sinensis, S. bovini, S. rommeli, S. bovifelis,*	Water, *N. caninum, T. gondii, Hammondia, other Sarcocystis* spp.
		Forward	S-18S-G4	5'- TTG AAT GAT CTA TCG CCA GC -'3	*S. fayeri, S. gigantea, S. bovifelis, S. hirsuta, S. buffalonis, S. bovini, S. sinensis, S. fusiformis*	
		Reverse	S-18S-G5	5'- AAC TTG AAT GAT CTA TCG CCA AT -'3	*S. tenella, S. cruzi, S. levinei*	
		Forward	S-18S-G6-F	5'- CAG TTA TAG TTT ATT TGA TAG TCA TC -'3	*S. tenella*	
		Reverse	S-18S-G6-R	5'- TCG CCA GCC ATT ACA AAA GA -'3		
		Reverse	S-18S-G9	5'- CAT CGC CGA CCA AAA AGG -'3	*S. neurona, S. lutrae*	
ITS1	External	Forward	NTH-ITS1-F	5'- CAT GAG YTT GYA TCT CTC T -'3	*N. caninum, T. gondii, and H. hammondia*	Water, *Sarcocystis* spp., *H. heydorni, H. triffittae*
		Reverse	NTH-ITS1-R	5'- TTT AGK AAG YAA TCT GAA AGC -'3		
	Internal	Forward	Neo-NP1	5'- TAC TAC TCC CTG TGA GTT G -'3	*N. caninum*	Water, *T. gondii, Sarcocystis* spp., *Hammondia* spp.
		Reverse	Neo-NP2	5'- TCT CTT CCC TCA AAC GCT -'3		
		Forward	Toxo-NP1	5'- GTG ATA GTA TCG AAA GGT AT -'3	*T. gondii, H. hammondia*	Water,*N. caninum, Sarcocystis* spp., *Hammondia* spp.
		Reverse	Toxo-NP2	5'- ACT CTC TCT CAA ATG TTC CT -'3		

2.2.3. Phylogenetic analysis

Phylogenetic analyses were performed on both the 18S rDNA and ITS1 sequences using MEGA6 software (Tamura *et al.*, 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (Tamura *et al.*, 2013). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, and all positions containing gaps and missing data were eliminated. The phylogeny was tested with the bootstrap method, using 1000 bootstrap replications. The 18S sequences were truncated at the 18S external primer site, whereas the ITS1 was truncated at both ends, ensuring all sequences started with the same homologous nucleotide positions.

2.2.4. PCR reactions and primer testing

TABLE 2. 3. PCR MASTER MIX SOLUTION FOR FIRST ROUND REACTION

Reagents	Volume (µl)
Primer F (10pmol/µl)	1
Primer R (10pmol/µl)	1
10X PCR Buffer	2
BioTaq TM DNA polymerase	0.15
Nuclease-free water	13.85
Template DNA	**2**
Total Master mix	**20**

Primers were used to amplify DNA from target sequences by nested PCR from *N. caninum, T. gondii* and various *Sarcocystis* spp. Primers were optimised using a temperature gradient (45^0C - 65^0C). Annealing temperatures were adjusted accordingly for each primer set. Once the primers were optimised, the best combination with the highest specificity and sensitivity was chosen.

Sensitivity was tested using a dilution series of *Neospora, Toxoplasma* and *Sarcocystis tenella* DNA. The dilution series consisted of 100ng/µl (10^{-1}), 1.0 ng/µl (10^{-2}), 0.1 ng/µl (10^{-3}), 0.01 ng/µl (10^{-4}), 0.001 ng/µl (10^{-5}). The first round PCR used the general outer primers, and the second round used the specific inner primer set (**Table 2. 2**).

The specificity was tested using protozoan control material acquired during this study (**Appendix Table II**). Each primer combination was run using at least one positive control and two negative controls (**Table 2. 2**). Samples, tested for protozoan DNA were tested in triplicates.

Briefly, each 20µl reaction contained 2µl of 10x custom PCR mix- (45mM Tris-HCl, 11mM $(NH_4)_2SO_4$, 4.5mM $MgCl_2$, 0.113mg/ml BSA, 4.4µM EDTA and 1.0mM each of dATP, dCTP, dGTP and dTTP) (ABgene, Epsom, Surrey, UK), 0.25pM of each primer (Eurofins MWG Operon), 0.75 units of BioTaq (Bioline, London, UK), 13.85µl of water and 2µl of sample DNA (**Table 2. 3**). The PCR conditions for the first round were 95°C for 5 mins followed by 35 cycles at 95°C for 1 min, 56°C for 1 min and 72°C for 1 min, with the final extension period at 72°C for 5 mins. The primary PCR amplicons were diluted with 100µl DNase / RNase free water, and 2µl of the diluted primary amplification product was added as template DNA for the second round amplification.

The reaction conditions for the second round PCR were identical to the first round, with the exception of an annealing temperature of 58°C for the internal 18S primers and 55°C for the internal ITS1 primers. Second round PCR products (10µl) were analysed by 2% agarose gel electrophoresis, stained with gel red (1:10, 1000 Biotonium, Hayward, USA) and visualised using UV light. Once primers were optimised, they were tested and validated using DNA from fixed and frozen tissues.

2.2.5. DNA extraction methods

DNA was extracted from fixed and frozen tissues, as per the QIAamp® DNA FFPE Tissue handbook for purification of genomic DNA from formalin-fixed, paraffin-embedded tissues and Wizard® Genomic DNA purification protocol. Following DNA purification from PCR amplicons, DNA was cloned into a pGEM-T easy plasmid, and plasmid DNA was purified via miniprep.

2.2.5.1. DNA extraction from formalin fixed paraffin was embedded (FFPE) tissues

Two x 10µm thick sections were cut from FFPE tissue blocks and placed into a sterile 1.5ml nucleic acid-free tube. New cutting blades and forceps were used for each tissue block, to avoid contamination. DNA was extracted using the QIAamp® DNA FFPE Tissue Kit (QIAGEN) according to the manufacturer's instructions. Briefly, 1ml of Xylene was added, vortexed for 10 seconds and centrifuged for 2 minutes at room temperature. The supernatant was removed, and 1ml of 96-100% ethanol was added to the pellets and mixed by vortexing, to extract any residual xylene from the sample. Samples were centrifuged at 10,000 g for 2 minutes, and the supernatant was removed. Pellets were incubated at room temperature for 10 minutes until the pellet became clear and all the ethanol had evaporated. The pellets were re-suspended and mixed in 360µl of buffer ATL and 35µl of proteinase K. The samples were incubated at 56°C overnight until samples had completely lysed. Samples were incubated at 90°C for one hour, to reverse the formaldehyde modification of nucleic acids. To each sample, 200µl of AL buffer and 200µl of 96-100% ethanol were added and mixed thoroughly by vortexing, until a white precipitate was formed. The samples were centrifuged, and the entire lysate was transferred to a QIAamp MiniElute column in a 2ml collection tube. Samples were washed with 500µl of Buffer AW1 and 500µl of Buffer AW2 and centrifuged for 1 minute each. After each wash, a new 2ml collection tube was used. Samples were centrifuged for 3 minutes to completely dry the membrane. The MiniElute columns were placed into a pre-labelled 1.5ml centrifuge tubes, 50-60µl of Buffer ATE was added and incubated at room temperature for 5 minutes. DNA was eluted and centrifuged for 1 minute. DNA concentrations (ng/µl) and purity (A260/230) were determined using the Nanodrop (see section 2.2.8. DNA quantification).

2.2.5.2. DNA extraction from frozen tissue

Approximately, 1g of each frozen-thawed tissue was transferred into a separate CK22 Precellys tissue homogenizer tube (Cepheid, Stretton Derbyshire, UK), containing CK28 ceramic beads and 1ml Nuclei Lysis Solution (Promega, Madison, WI, USA).

Samples were homogenized for 2 x 50s at 5000 g using a Precellys 24 tissue homogenizer (Depheid, Stretton Derbyshire, UK). Around 400µl of homogenised tissue was added to 900µl of nuclei lysis solution and incubated at 55°C overnight. Samples were processed using the Wizard® Genomic DNA (Promega, Madison WI, USA) purification protocol, which was adapted to use 0.4g of starting material (Bartley *et al.*, 2013; Lepore *et al.*, 2017). DNA samples were stored at -20°C.

2.2.5.3. DNA purification and PCR clean-up system

PCR products were purified using the Promega Wizard SV gel and PCR clean-up system (Promega, UK) according to manufacturer's instructions. Briefly, either the correct band was cut from the agarose gel, weighed and dissolved at 56°C using 10µl of Membrane Binding Solution per 10mg of gel. Alternatively, the PCR product was cleaned, and an equal volume of Membrane Binding Solution was added. The gel mixture or the prepared PCR product were transferred into the Minicolumn assembly (SV Minicolumn and collection tube), incubated at room temperature for 1 minute and centrifuged at 16,000 g for 1 minute. The Minicolumn, containing the bound DNA, was washed twice with Membrane Wash Solution (700µl and 500µl) and centrifuged at 16,000 g for 5 minutes each. The Minicolumn was centrifuged for 1 minute at 16,000 g to allow evaporation of any residual ethanol. DNA was eluted by incubating 50µl of nuclease-free water at room temperature for 1 minute. DNA was stored at –20°C. The nucleic acid concentration was determined by spectrophotometer (Nanodrop ND1000) (see section 2.2.8. DNA quantification).

2.2.6. Cloning of target DNA

Target products (inserts) were cloned using the pGEM®-T Easy Vector System (Promega, Madison, WI, USA) as per Lepore *et al.* (2017). Briefly, 2 microliters (64ng) of the purified product was ligated into the pGEM®-T Easy Vector (1µl at 50ng/µl) using 1µl of T4 DNA ligase (3 Weiss units/µl) and 5µl of 2x Rapid Ligation Buffer. Following ligation, 1µl of ligated vector / insert was used to transform 40µl of high-efficiency competent JM109 cells (≥1 × 108cfu/µg DNA) (Promega, Madison, WI, USA) using manufacturer's instructions, with the exception of using LB-broth

medium instead of SOC medium to culture the bacteria. A successful transformation was confirmed using prepared LB agar plates containing 100µg/ml ampicillin, spread with 100µl of Isopropyl-β-D-thiogalactopyranoside (IPTG) of 100mM and 20µl of X-Gal (50mg/ml) which allows for blue / white selection. The pGEM-T-Easy plasmid contains the β-galactosidase enzyme gene. When an unsuccessful transformation occurred, the β-galactosidase enzyme gene is functional, and the enzyme metabolises X-Gal resulting in a blue colouration of the colonies. A successful transformation in *E. coli* was confirmed with white colonies. White colonies were screened by colony PCR using the primers designed for each target insert to confirm the correct target insert. Clones containing the correct sequence / target insert were grown overnight in 10ml bottles of containing LB and 100µg/ml ampicillin.

2.2.6.1. Miniprep of plasmid DNA

The plasmids were purified using QIAprep® Spin Miniprep Kit (QIAGEN) as per manufacturer's instructions. Briefly, 10 ml of the overnight culture was pelleted at 13,000 g at room temperature for 5 minutes. The bacterial pellet was re-suspended in 250µl buffer P1 (50mM Tris pH 8.0, 10mM EDTA, 100µglml RNase A). First, 250µl of cell lysis buffer P2 (200mM NaOH, 1% SDS), then 350µl of buffer N3 was added and mixed by inverting the tube 4-6 times until precipitation occurred. The tubes were centrifuged for 10 minutes at 13,000 g to pellet the precipitation. Roughly, 800µl of the supernatant was added to the QIAprep 2.0 spin column, centrifuged for 30-60 seconds and the flow-through discarded. The spin columns were washed by adding 0.5ml of buffer PB to remove trace nuclease activity when using endA+ strains, such as JM09, and centrifuged for 30-60 seconds. The column was washed with 0.75ml of buffer PE and centrifuged for 30-60 seconds. The flow-through was discarded and the column centrifuged for an additional 1 minute to remove any residual wash buffer. The columns were placed into a clean 1.5ml microcentrifuge tube, and the plasmid DNA was eluted using 50µl EB elution buffer (10mM Tris. Cl, pH 8.5) by centrifugation.

2.2.7. Generation of control DNA using glycerol stocks

A 30% glycerol stock was made by adding 200µl glycerol and 800µl bacteria of each overnight culture containing the target insert. Samples were stored at -80^{0}C. Each clone was streaked out on a new set of agar plates, and one colony was picked using a P20 pipette tip and transferred in 1 ml of dH_2O. The colony clone was heated to 95^{0}C to release DNA. Plasmid DNA was diluted to 10^{-3} and 10^{-6} and was ready to be used to validate PCR primers.

2.2.8. DNA quantification

The DNA concentration and purity were determined by a Nanodrop ND1000 spectrophotometry (Labtech International Ltd, Uckfield, East Sussex, UK). The absorbance of 260nm and purity of a ration between 260nm and 280nm was measured.

2.2.9. Sequencing and sequencing analysis

DNA was sequenced using the Eurofins MWG Operon sequencing service. Purified genomic or plasmid DNA were sent for sequencing at a concentration of 100ng per sample using the appropriate forward and reverse primers at a concentration of 5pmol/µl, to verify the sequence and determine which protozoal species was present. Plasmid clones were sent for sequencing using the T7 and SP6 primers provided by MWG. Sequences were analysed using the DNASTAR SeqMan Pro^{TM} software. A consensus sequence was generated from the forward and reverse sequences, and BLAST searched using the NCBI database to verify the correct region, gene, insert, species was amplified / cloned. The percentage identity and query cover were taken, and the sequence was compared to other published sequences. If a sequence had not been identified, it was submitted to GenBank. Clones were checked to ensure that no base pair changes or PCR errors had occurred. Clones where errors in the base pair sequence occurred, could affect the amino acid composition, and so a different colony was used.

2.2.10. Submission of sequences to GenBank

Sequences were submitted to GenBank BankIT (https://www.ncbi.nlm.nih.gov/WebSub/?form=history&tool=genbank), and accession numbers were completed in accordance.

2.2.11. PCR primers validation

For the validation of PCR primers, samples from different host animals (cattle, sheep, horses, carnivores, birds and whale) were tested and a total of 98 fixed tissue and 81 fresh tissue samples were tested (**Appendix table IV A**). Fresh tissues were frozen to -20°C following full necropsies and DNA from frozen tissue was extracted as per section 2.2.5.2. For fixed tissues samples, one block per animal case was selected and DNA from fixed tissue samples was extracted as per section 2.2.5.1. PCR reactions were performed as described in 2.2.4. PCR reactions and primer testing. Positive PCR products were purified as per 2.2.5.3. DNA purification and PCR clean-up system and sent for sequencing as per 2.2.9. Sequencing and sequencing analysis.

First tests were performed on fixed samples (n= 82) from 61 cattle and 21 sheep and were tested for protozoan DNA (**Appendix table IV A**). Samples were received from the surveillance archives from Moredun Pathology and samples were selected based on their availability, complete data sets and previous IHC analysis of findings of lesions or cysts consistent with protozoal infections in brain, placenta or muscle tissues from aborted foetuses. Results were compared from the Moredun Pathology data base using the previous IHC results (with the Moredun In-house antisera **Table 4.1**).

Next tests, 54 European badger (*M. meles*) carcasses were collected from around the Lothians and Borders regions of Scotland, following fatal collisions with vehicles (badgers were collected with the knowledge and permission of Scottish Natural Heritage) (Bartley *et al.*, 2013; Burrells *et al.*, 2016) (**Appendix table IV B**). Carcasses were stored at -20°C prior to processing, full necropsies were performed when possible where samples of neck muscle, tongue, spleen, submandibular lymph node, liver, lung, brain, heart, blood and spinal cord were collected. Full information available in the study by Bartley *et al.*, (2013). DNA from fresh tissue samples was extracted, and PCR

reactions were performed as described by Lepore *et al.* (2017). Positive PCR products were purified as per 2.2.5.3. DNA purification and PCR clean-up system, and sent for sequencing as per 2.2.9. Sequencing and sequencing analysis. For full methodology see Lepore *et al.* (2017).

Last tests, 27 fresh tissue and 16 fixed samples were tested via PCR for protozoan DNA that were sent to Moredun pathology (**Appendix table IV A and B**). A total of 13 sheep (9 fixed and 4 fresh) and 9 bovine (3 fresh and 6 fixed) that were sent to Moredun pathology were selected based on clincal presentations from aborted foetal tissue (brain, spinal cord and placenta) suspected of protozoan infections (5 ovine and 3 bovine) and based on previous findings of lesions or cysts consistent with protozoal infections in muscle tissue from previous histopathology analysis (8 ovine and 6 bovine). Moreover fresh tissues from 2 cappercailies, n= 1 mink, n= 2 pine marten, n= 1 bat, n= 1 whale and n= 14 horse) from muscle and heart tissue that were sent to Moredun pathology based on previous findings of lesions or cysts consistent with protozoal infections, were selected (**Appendix table IV B**).

Primers were generally used as follows:

First, samples were tested using general protozoan primers (I and II) and general *Neospora* and *Toxoplasma* primers (III). If the PCR results came back negative, samples were assumed to be negative for protozoal DNA.

I. External primers NTS-18S-F1 and NTS-18S-R1
II. Internal primers NTS-18S-F2 and NTS-18S-R2
III. Internal primers NTH-18S-F2 and NTH-18S-R2

If samples come back positive for the 18S general primer and negative for the 18S general *Neospora* and *Toxoplasma* primer set, they were assumed negative for either *Neospora* or *Toxoplasma*. Samples were tested for *Sarcocystis* spp. with *Sarcocystis* group primers (I, IV, V, VI, VII, VIII, IX, X). Positive PCR amplicons were sent for sequencing for confirmation of the *Sarcocystis* species. The *Sarcocystis* primer sets were used depending on which animal host species the samples came from; i.e. ovine or bovine.

IV. Internal primer S-18S-G1-F and NTS-18S-R2
V. Internal primer S-18S-G2-F and NTS-18S-R2
VI. Internal primer S-18S-G3-F and NTS-18S-R2
VII. Internal primer NTS-18S-F2 and S-18S-G4-R
VIII. Internal primer NTS-18S-F2 and S-18S-G5-R
IX. Internal primer S-18S-G6-F2 and S-18S-G6-R2
X. Internal primer S NTS-18S-F2 + S-18S-G9

If samples were positive for the 18S general primer and positive for the 18S general *Neospora* and *Toxoplasma* primers, they were further tested using the ITS1 *Neospora* specific (XI and XII) and *Toxoplasma* specific primers (XI and XIII). Positive PCR amplicons were sent for sequencing for confirmation of *Neospora* / *Toxoplasma* DNA. External primers NTH-ITS1-F1 and NTH-ITS1-R1

XI. Internal primers Neo-NP1 and Neo NP2
XII. Internal primers Toxo-NP1 and Toxo-NP2

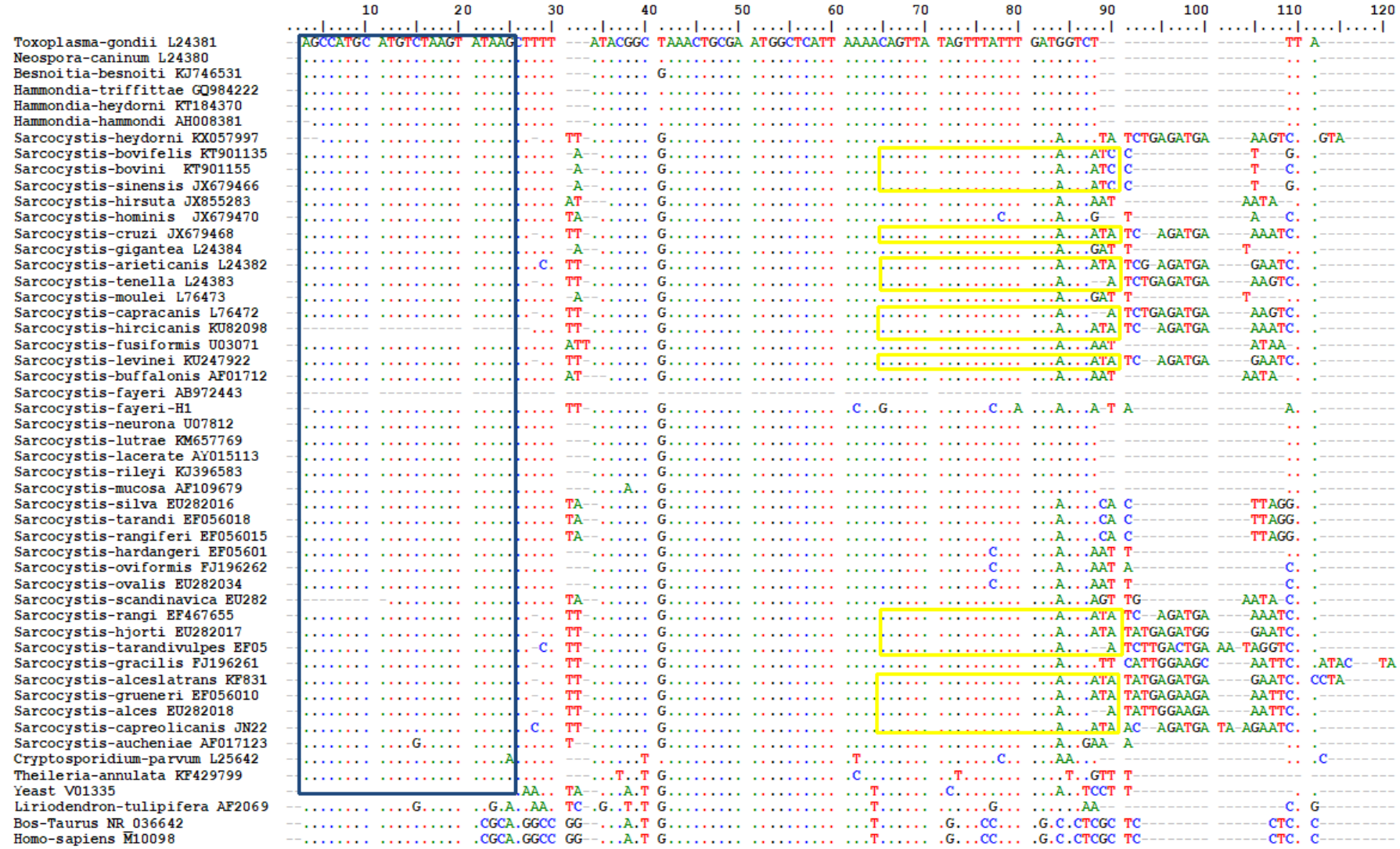
2.2.12. Statistical analysis

The samples prevalence was calculated with confidence intervals of 95% CI for the presence of positive and negative samples. All of the calculations were carried out using Minitab 17 software (v17.1.0.0).

Chapter 2.3. Results

2.3.1. Identification and development of general pan 18S primers

Primers were designed using a sequence alignment tool which highlights base pair changes between group specific primers (**Figure 2. 1**). The multiple sequence alignment shows relatively high polymorphism within the 18S sequences able to distinguish between *Neospora*, *Toxoplasma* and *Sarcocystis* spp.. Five 18S general outer primers that amplify Apicomplexa species, including *Neospora*, *Toxoplasmas*, *Hammondia* spp., and various *Sarcocystis* spp., were designed from a region that was conserved for protozoan species, yet showed enough polymorphism from the host species and other closely related species (**Figure 2. 1**). For each outer primer designed, a minimum of 1 base pair change to a maximum of 9 base pair differences were observed for host species, unrelated species *L. tulipifera* and *C. parvum*, *T. annulata* and *Yeast* (**Figure 2. 1**). Two forward and three reverse outer primers were designed creating six different outer primers combinations. Based on *in silico* analysis, species amplified by each primer are represented in **Table 2. 4**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Sarcocystis-rileyi KJ396583
Sarcocystis-mucosa AF109679
Sarcocystis-silva EU282016
Sarcocystis-tarandi EF056018
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Sarcocystis-hardangeri EF05601
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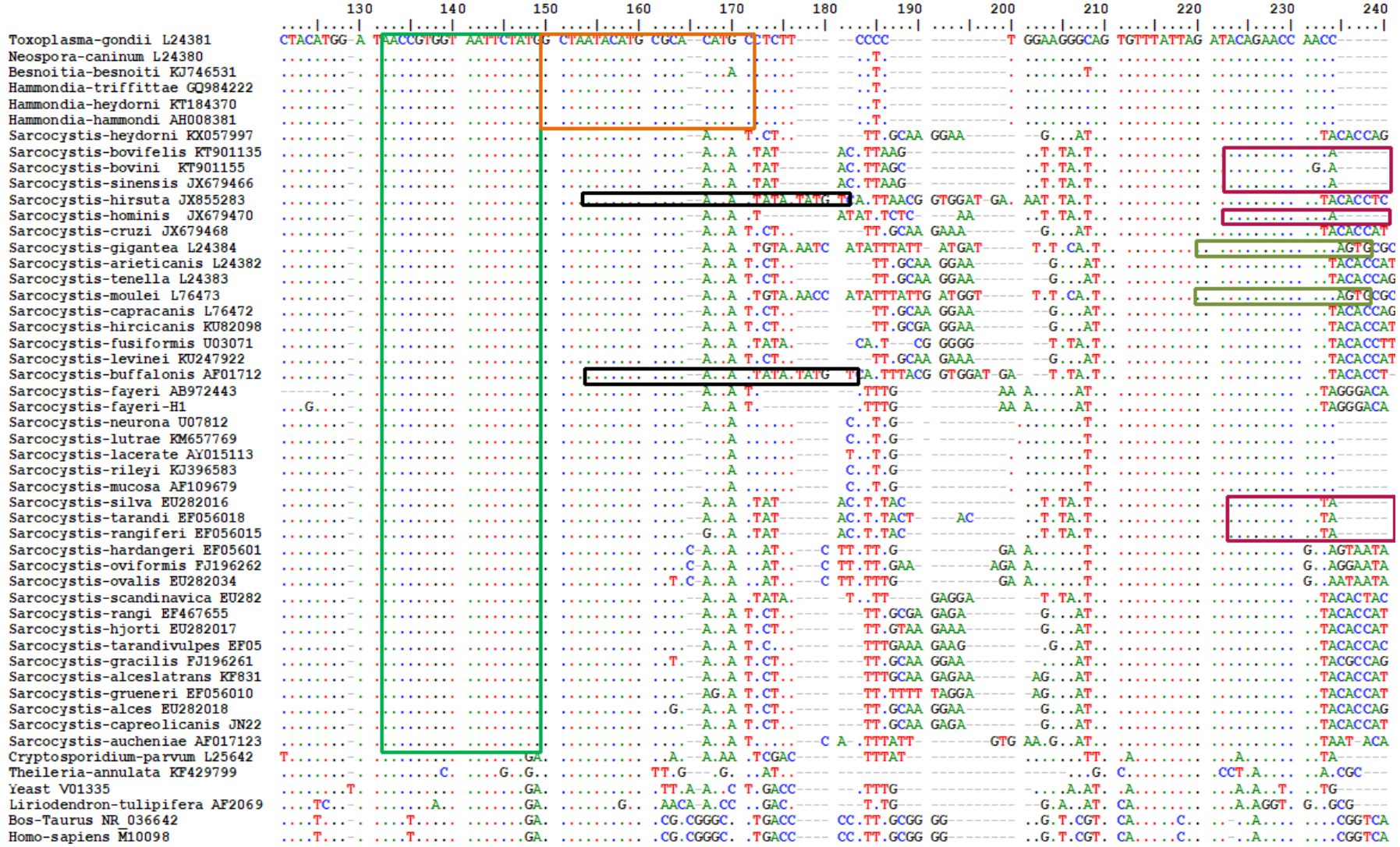
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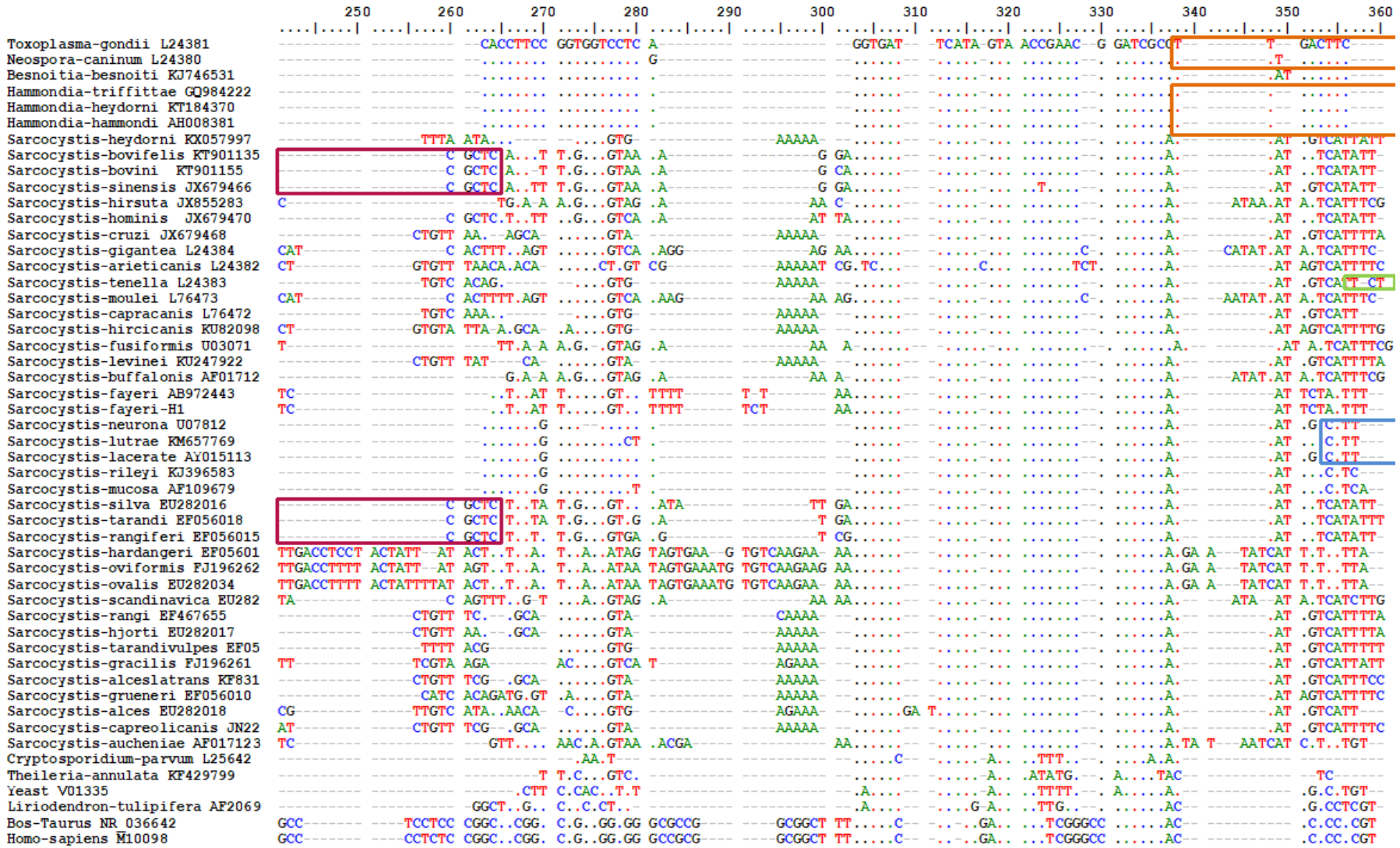
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Sarcocystis-oviformis FJ196262
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Liriodendron-tulipifera AF2069
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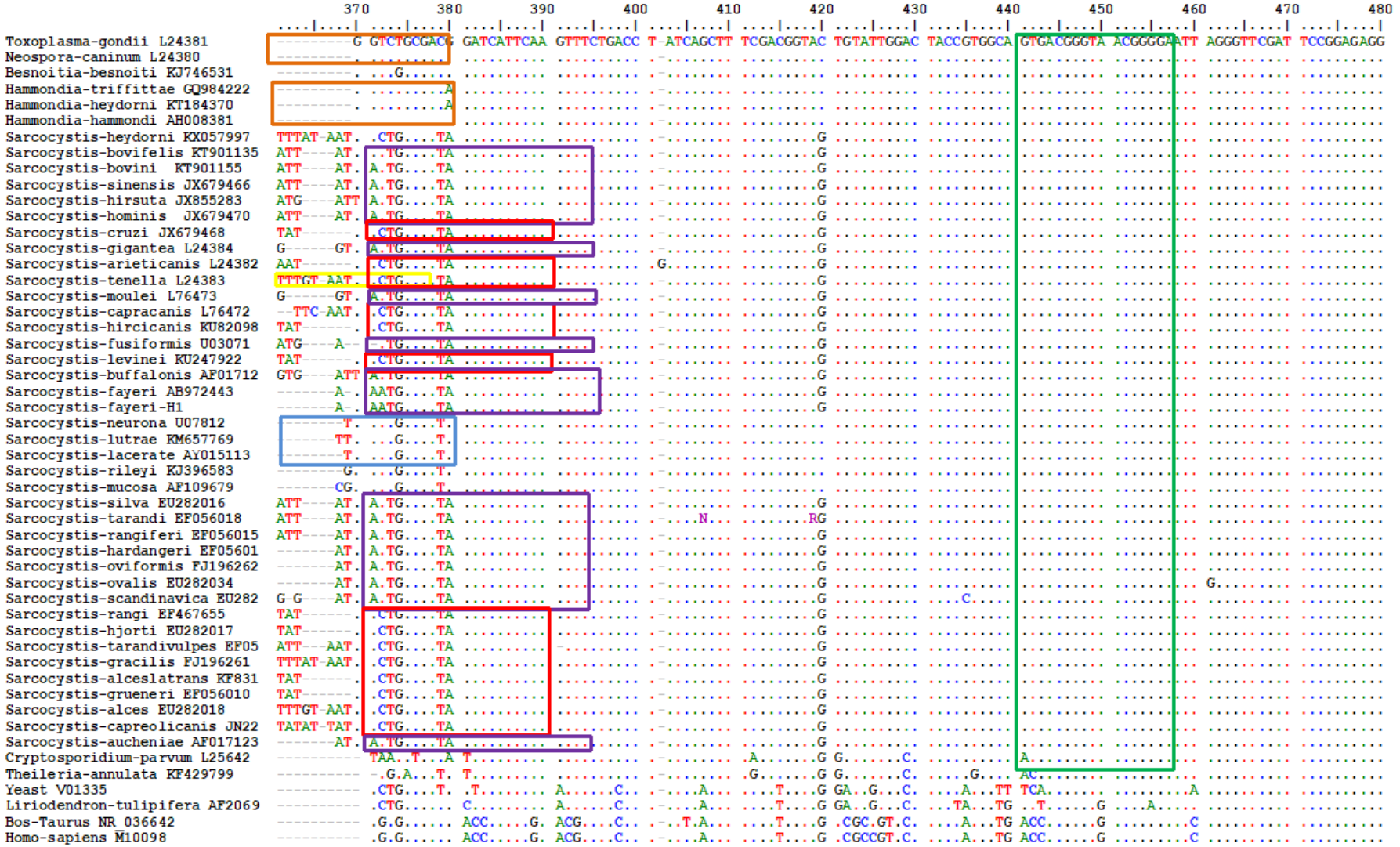
Homo-sapiens M10098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Besnoitia-besnoiti KJ746531
Hammondia-triffittae GQ984222
Hammondia-heydorni KT184370
Hammondia-hammondi AH008381
Sarcocystis-heydorni KX057997
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Sarcocystis-bovini KT901155
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Sarcocystis-hominis JX679470
Sarcocystis-cruzi JX679468
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Sarcocystis-moulei L76473
Sarcocystis-capracanis L76472
Sarcocystis-hircicanis KU82098
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Sarcocystis-levinei KU247922
Sarcocystis-buffalonis AF01712
Sarcocystis-fayeri AB972443
Sarcocystis-fayeri-H1
Sarcocystis-neurona U07812
Sarcocystis-lutrae KM657769
Sarcocystis-lacerate AY015113
Sarcocystis-rileyi KJ396583
Sarcocystis-mucosa AF109679
Sarcocystis-silva EU282016
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Sarcocystis-rangiferi EF056015
Sarcocystis-hardangeri EF05601
Sarcocystis-oviformis FJ196262
Sarcocystis-ovalis EU282034
Sarcocystis-scandinavica EU282
Sarcocystis-rangi EF467655
Sarcocystis-hjorti EU282017
Sarcocystis-tarandivulpes EF05
Sarcocystis-gracilis FJ196261
Sarcocystis-alceslatrans KF831
Sarcocystis-grueneri EF056010
Sarcocystis-alces EU282018
Sarcocystis-capreolicanis JN22
Sarcocystis-aucheniae AF017123
Cryptosporidium-parvum L25642
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Yeast V01335
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Homo-sapiens M10098

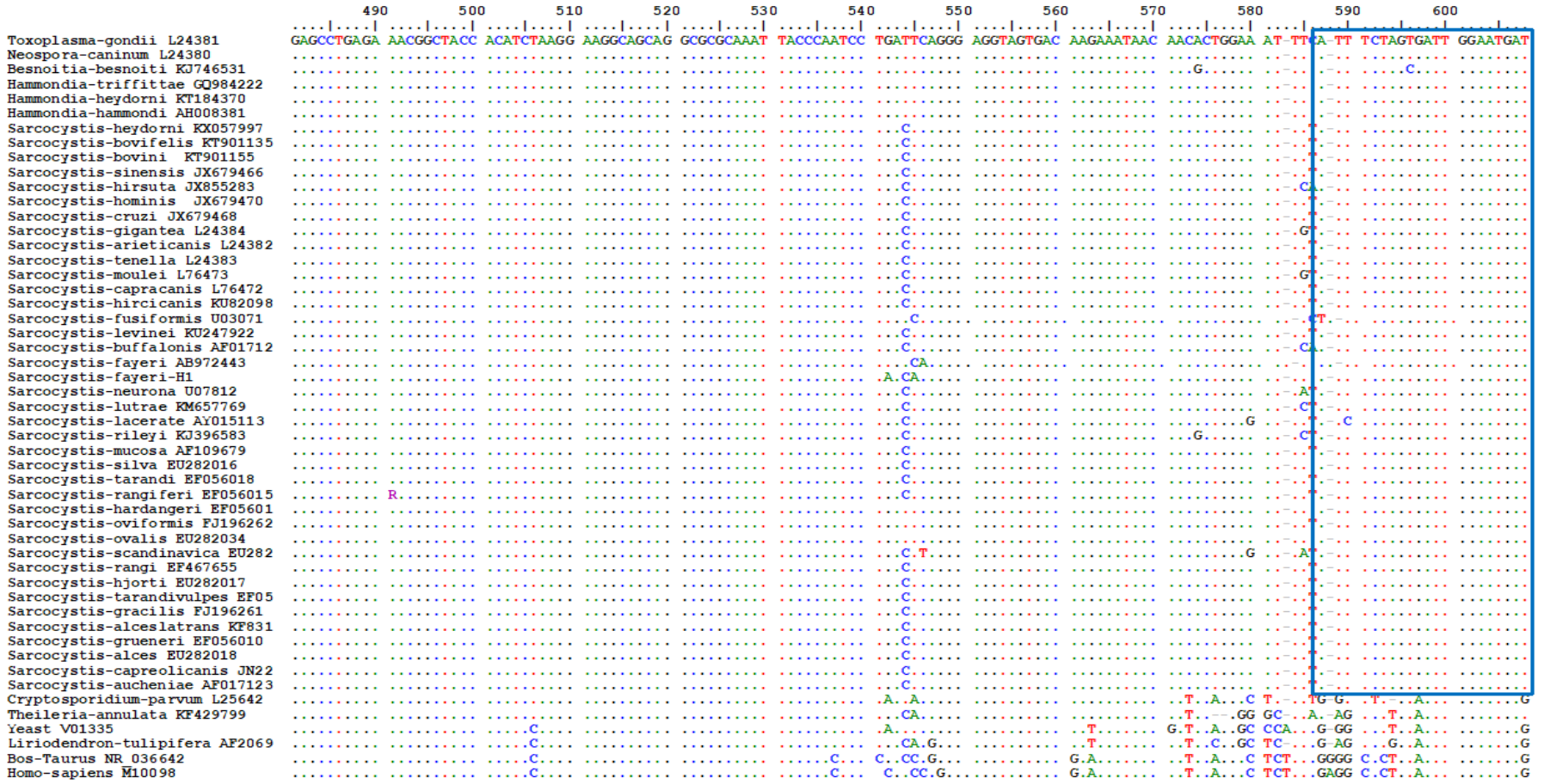


FIGURE 2. 1. A SEQUENCE ALIGNMENT OF THE 18S RRNA GENE OF ALL PROTOZOAN SPECIES INCLUDED IN THIS STUDY GENERATED FROM THE OUTER PRIMER SETS 'NTS-18S-F1 AND NTS-18S-R1'. DASHES REPRESENT IDENTICAL BASE PAIR CHANGE, AND DOTS REPRESENT GAPS IN THE ALIGNMENTS. EACH COLOURED BOX REPRESENTS A PRIMER DESIGNED FROM THE ALIGNMENT. PRIMERS ARE REPRESENTED BY THE FOLLOWING COLOURS; DARK BLUE - 'NTS F1/R1', DARK GREEN – 'NTS F1/R1', ORANGE- 'NTH', LIGHT GREEN- 'G6', BLACK- 'G2', PINK- 'G3', PURPLE- 'G4', RED- 'G5', YELLOW- 'G6', LIGHT BLUE- 'G9'.

PCR results showed that all general 18S outer primer combinations worked at an optimum temperature of 56°C (**Figure 2. 2**). *Neospora*, *Toxoplasma* and *Sarcocystis* DNA was amplified up to 0.01ng/µl **(Figure 2. 3).** The outer primer set NTS-18S-F1 + NTS-18S-R1, NTS-18S-F1 + NTS-18S-R2, NTS-18S-F2 + NTS-18S-R1, NTS-18S-F2 + NTS-18S-R2, and NTS-18S-F2 + NTS-18S-R3 showed successful amplification of *T. gondii, N. caninum, Hammondia* spp. and various *Sarcocystis* spp. based on PCR and *in silico* analysis (**Table 2. 4** and **Figure 2. 1**). No amplification of other closely related (*Cryptosporidium*, *Babesia* or *Theleria*) and host species (bovine and ovine) was observed.

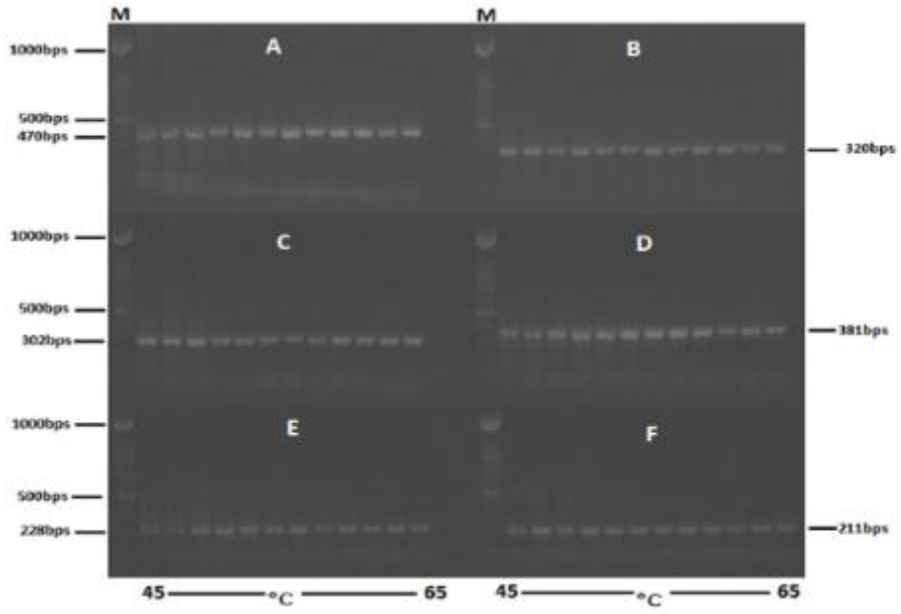


FIGURE 2. 2 OPTIMISATION OF SIX APICOMPLEXA PAN OUTER PRIMER SETS USING TEMPERATURE GRADIENTS, FROM 45 °C TO 65°C FOR THE DETECTION OF *N. CANINUM* DNA. M REPRESENTS A 100 BASE PAIR HYPER LADDER MARKER (BIOLINE). PRIMER SET A= NTS-18S-F1 + NTS-18S-R1, PRIMER SET B= NTS-18S-F1 + NTS-18S-R2, PRIMER SET C= NTS-18S-F1 + NTS-18S-R3, PRIMER SET D= NTS-18S-F2 + NTS-18S-R1, PRIMER SET E= NTS-18S-F2 + NTS-18S-R2 AND PRIMER SET F= NTS-18S-F2 + NTS-18S-R3.

TABLE 2. 4. REPRESENTATIVE OUTER (GENERAL) AND INNER (SPECIFIC) PRIMERS DESIGNED FROM THE 18S RRNA GENE AND SPECIES THAT ARE MOST LIKELY TO BE AMPLIFIED BASED IN SILICO ANALYSIS

Forward/ Reverse	Product name	Species detected
	General	
F	NTS-18S-F1	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsuta, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, S. heydorni, S. bovini, S. hominis, S. hircicanis, S. fayeri, S. levinei, H. hammondia, H. heydorni, H. triffittae, Theileria anulata, C. parvum, Yeast,*
F	NTS-18S-F2	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsuta, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, S. heydorni, S. bovini, S. hominis, S. hircicanis, S. fayeri, S. levinei, H. hammondia, H. heydorni, H. triffittae*
R	NTS-18S-R1	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsuta, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, S. heydorni, S. bovini, S. hominis, S. hircicanis, S. fayeri, S. levinei, H. hammondia, H. heydorni, H. triffittae*
R	NTS-18S-R2	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsuta, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, S. heydorni, S. bovini, S. hominis, S. hircicanis, S. fayeri, S. levinei, H. hammondia, H. heydorni, H. triffittae, C. parvum*
R	NTS-18S-R3	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsuta, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, S. heydorni, S. bovini, S. hominis, S. hircicanis, S. fayeri, S. levinei, H. hammondia, H. heydorni, H. triffittae*
	Specific	
F	S-18S-G1	*S. gigantea, S. moulei*
F	S-18S-G2	*S. hirsuta, S. buffalonis*
F	S-18S-G3	*S. rangiferi, S. tarandi, S. sinensis, S. silvia, S. bovini, S. hominis*
R	S-18S-G4	*S. silva, S. tarandi, S. sinensis, S. rangiferi, S. fusiformis, S. moulei, S. gigantea, S. scandinavica, S. oviformis, S. ovalis, S. fayeri, S. hardangeri, S. buffalonis, S. hirsuta, S. hominis and S. bovini*
R	S-18S-G5	*S. tenella, S. arieticanis, S. grueneri, S. alces, S. capreolicanis, S. gracilis, S. alceslatrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. heydorni and S. hircanis,*
F/R	S-18S-G6	*S. tenella*
R	S-18S-G9	*S. neurona, S. lutrae, S. lacerate, S. rileyi*

The outer primers NTS-18S-F1 only showed 0 to 1 base pair difference to the closely related species *T. anulata, C. parvum, Yeast* whereas 3 to 4 base pair difference was observed towards other unrelated and host species (**Figure 2. 1)**. However, based on *in silico* analysis when these primers are used in combination with NTS-18S-R1, NTS-18S-R3 and NTS-18S-F2 respectively, amplification of *T. anulata, C. parvum* and *Yeast* were eliminated. The exception was primers NTS-18S-F1 + NTS-18S-R2, which showed amplification of *C. parvum* based on *in silico* analysis and PCR results. PCR results that showed that each primer combination displayed no difference in sensitivity and was shown to be specific i.e. only amplify protozoan species *Neospora, Toxoplasma* and *Sarcocystis*. The external primer combination NTS-18S-F1 + NTS-18S-R1 was chosen and taken forward for further studies.

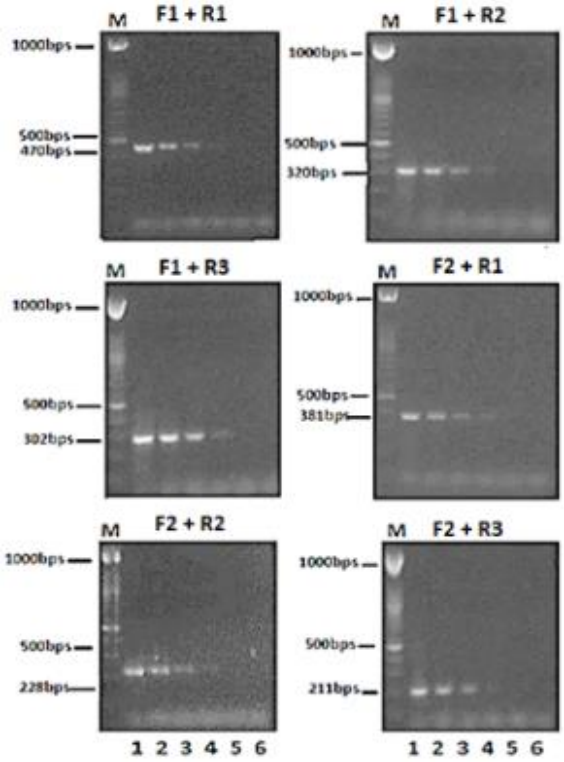


FIGURE 2. 3. DILUTION PCR USING THE SIX APICOMPLEXA GENERAL OUTER PRIMERS AT 56°C DURING THE ANNEALING STAGE, TO TEST THE SENSITIVITY OF *N. CANINUM* DNA (NEO1 AND NEO2). M REPRESENTS A 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- 1/10 DILUTION, 2- 1/100 DILUTION, 3- $1/10^{-3}$ DILUTION, 4- $1/10^{-4}$ DILUTION, 5- $1/10^{-5}$ DILUTION, AND 6- NEGATIVE CONTROL (H_2O).

2.3.2 Identification and development of the 18S *Neospora and Toxoplasma* primers

Three sets of primers that specifically detect *N. caninum* and *T. gondii* DNA were designed (**Table 2. 5**). Each genus-specific primer set was tested using a temperature gradient, and an optimum of 62°C for N-18S-F2 + N-18S-R2 and T-18S-F2 + T-18S-R2 and 58°C for NTH-18S-F2 + NTH-18S-R2 was chosen. The primer set N-18S-F2 + N-18S-R2 amplified *N. caninum* DNA; primer set T-18S-F2 + T-18S-R2 amplified *T. gondii* DNA (data not shown). Primer set NT-18S-F2 + NT-18S-R2 successfully amplified *Neospora*, *Toxoplasma* and *Hammondia* spp. DNA (**Figure 2. 5 A**). The sequence alignment demonstrated that *T. gondii, N. caninum, H. hammondia, H. heydorni, H. triffittae and B. besnoiti* showed limited polymorphism within the 18S region compared to the *Sarcocystis* spp. (**Figure 2. 1**). Only a few base pair differences were observed between *Neospora, Toxoplasma* and the *Hammondia* spp. (**Figure 2. 1**). A low degree of polymorphism in the 18S rRNA gene between *Neospora, Toxoplasma* and *Hammondia* spp. was observed in the phylogenetic tree (**Figure 2. 4**).

The results showed that the primer set N-18S-F2+ N-18S-R2 specifically designed for *Neospora,* also showed amplification of *Toxoplasma* DNA (data not shown). The primer set T-18S-F2+ T-18S-R2, specifically designed for *Toxoplasma*, also showed amplification of *Neospora* DNA (data not shown). The primer set NTH-18S-F3+ NTH-18S-R3 showed specific amplification of *T. gondii, N. caninum, H. triffittae, H. heydorni,* and *H. hammondia* DNA and no amplification of *Sarcocystis* DNA was observed (**Figure 2. 5 A**).

TABLE 2. 5. REPRESENTATION OF EACH *NEOSPORA* AND *TOXOPLASMA*-SPECIFIC PRIMER COMBINATION FROM THE 18S AND ITS1 REGION, INCLUDING SPECIES AMPLIFIED BY EACH PRIMER SET BASED ON *IN SILICO* ANALYSIS.

Forward Primer	Species specificity	Reverse Primer	Species specificity	Primer combination	Combination specificity	Base pairs (bps)
***Neospora* / *Toxoplasma* primers**						
N-18S-F2	*N. caninum*	N-18S-R2	*N. caninum*	N-18S-F2+ N-18S-R2	*N. caninum*	~120
T-18S-F2	*T. gondii*	T-18S-R2	*T. gondii*	T-18S-F2+ T-18S-R2	*T. gondii*	~117
NTH-18S-F2	*N. caninum*, *T. gondii*, *H. hammondia*, *H. heydorni*, *H. triffittae*,	NTH-18S-R2	*N. caninum*, *T. gondii*, *H. hammondia*, *H. heydorni*, *H. triffittae*	NT-18S-F2+ NT-18S-R2	*N. caninum*, *T. gondii*, *H. hammondia*, *H. heydorni*, *H. triffittae*	~130
NT-ITS1-F1	*N. caninum*, *T. gondii*, *H. hammondia*, *H. heydorni*, *H. triffittae*	NT-ITS1-R1	*N. caninum*, *T. gondii*, *H. hammondia*	NT-ITS1-F+ NT-ITS1-R	*N. caninum*, *T. gondii*, *H. hammondia*	~355/ 330
Neo-NP1 (ITS1)	*N. caninum*	Neo-NP2 (ITS1)	*N. caninum*	Neo NP1+ Neo NP2	*N. caninum*	~249
Toxo-NP1 (ITS1)	*T. gondii*, *H. hammondia*	Toxo-NP2 (ITS1)	*T. gondii*, *H. hammondia*	ToxoNP1+ Toxo NP2	*T. gondii*, *H. hammondia*	~227

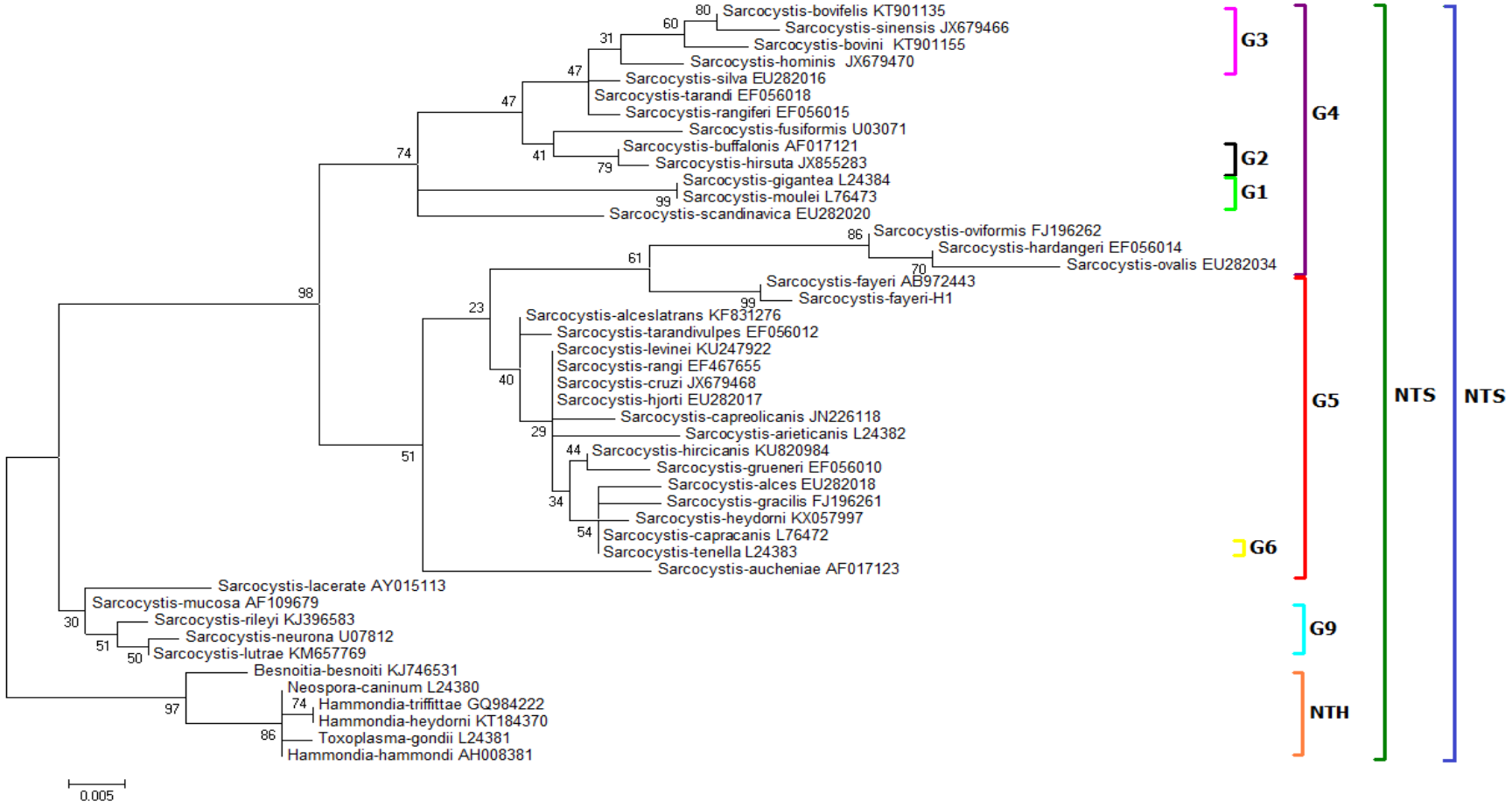


FIGURE 2. 4. 18S PHYLOGENETIC TREE SHOWING THE DIVERSITY AND COMPLEXITY OF THE PROTOZOAN SPECIES: *SARCOCYSTIS* SPP., *TOXOPLASMA* AND *NEOSPORA*. DEVELOPED PRIMERS ARE HIGHLIGHTED IN BRACKETS. DARK BLUE- 'NTS F1/R1', DARK GREEN– 'NTS F1/R1', ORANGE- 'NTH', LIGHT GREEN- 'G6', BLACK- 'G2', PINK- 'G3', PURPLE- 'G4', RED- 'G5', YELLOW- 'G6', LIGHT BLUE- 'G9'.

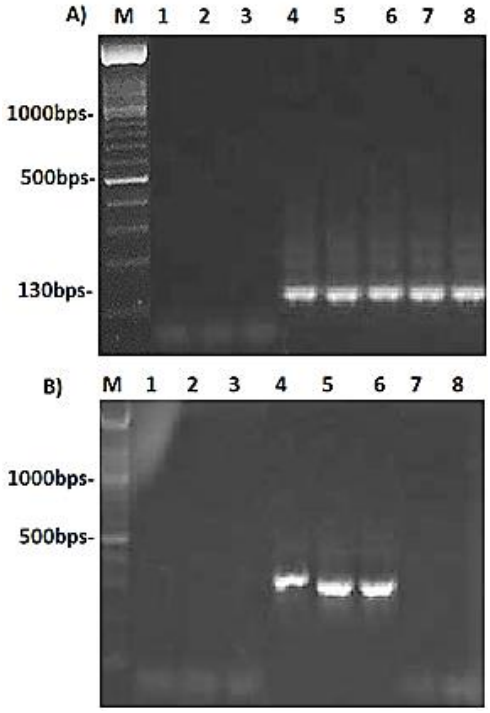


FIGURE 2. 5. PCR TO TEST THE SPECIFICITY OF A) NTH-18S-F2 + NTH-18S-R2. M REPRESENTS 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- *S. LUTRAE*, 2- *S. TENELLA*, 3- dH_2O., 4- *NEOSPORA* AND 5- *TOXOPLASMA*, 6- *H. HAMMONDIA*, 7- *H. HEYDORNI*, 8- *H. TRIFFITTAE*. B) NT-ITS1-F1 + NT-ITS1-R1. M REPRESENTS 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- dH_2O, 2- *S. TENELLA*, 3- *S. NEURONA*, 4- *TOXOPLASMA*, 5- *N. CANINUM*, 6- *H. HAMMONDIA*, 7- *H. HEYDORNI*, 8- *H. TRIFFITTAE*.

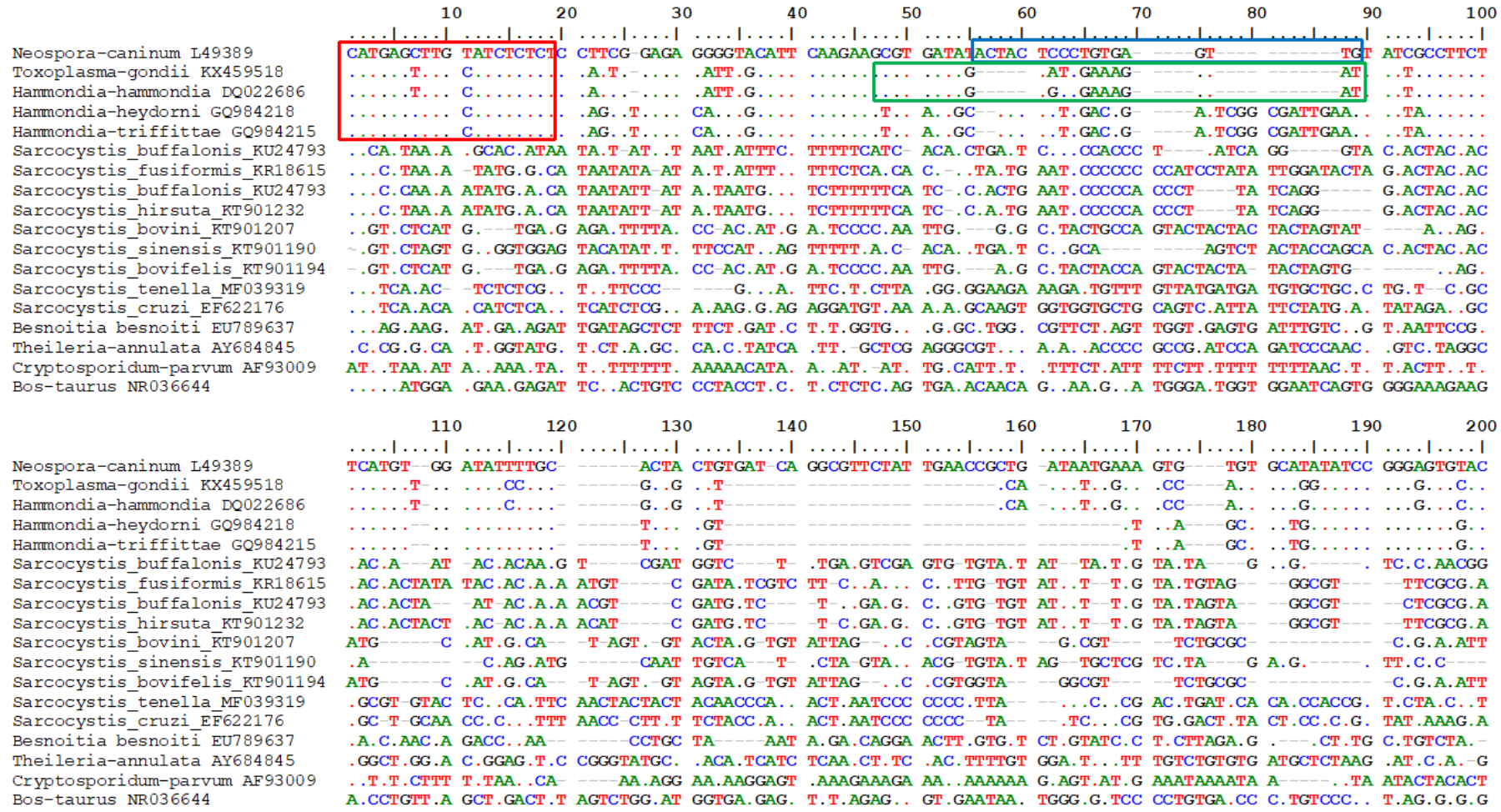
2.3.3. Identification and development of the ITS1 *Neospora* and *Toxoplasma* primers

Two general outer primers for the ITS1 sequence were designed to specifically amplify DNA from both *Toxoplasma* and *Neospora* (**Table 2. 5**). From the ITS1 sequence alignment, it can be seen that the outer forward primer 'NT-ITS1-F1' based on *in silico* analysis should amplify *N. caninum, T. gondii, H. hammondia, H. heydorni, H. triffittae, and B. besnoiti*, whereas the outer reverse primer 'NT-ITS1-R1' showed more specificity and should only amplify the species *N. caninum, T. gondii, H. hammondia* (**Figure 2. 6**). When both outer primers are used, the species *N. caninum, T. gondii, H. hammondia* should be amplified based on *in silico* analysis.

ITS1 outer primers were optimised in a temperature gradient, and an optimum temperature of 56°C was chosen. ITS1 outer primers successfully amplified *Neospora* at 330 bp and *Toxoplasma* at 355 bp, *H. hammondia* at 328 bp (**Figure 2. 5 B**). A

Sensitivity test of the ITS1 PCR showed amplification up to a dilution of 0.1ng/µl (data not shown). PCR results showed amplification of *Neospora*, *Toxoplasma* and *H. hammondia* DNA, yet no *H. heydorni*, *H. triffittae* or *Sarcocystis* spp. DNA was amplified (**Figure 2. 5 B**).

An alignment for the ITS1 region was performed to highlight polymorphic and conserved regions within this region of the primer design (**Figure 2. 6**). The ITS1 sequence alignment showed a higher polymorphism compared to the 18S region for the species *Neospora*, *Toxoplasma* and *Hammondia* spp. (**Figure 2. 6**). The ITS1 phylogenetic analysis also demonstrated a higher differentiation between *Neospora*, *Toxoplasma* and *Hammondia* spp. compared to the 18S rRNA gene (**Figure 2. 7**). A higher diversity among closely related species was observed (**Figure 2. 7**).


Neospora-caninum L49389
Toxoplasma-gondii KX459518
Hammondia-hammondia DQ022686
Hammondia-heydorni GQ984218
Hammondia-triffittae GQ984215
Sarcocystis_buffalonis_KU24793
Sarcocystis_fusiformis_KR18615
Sarcocystis_buffalonis_KU24793
Sarcocystis_hirsuta_KT901232
Sarcocystis_bovini_KT901207
Sarcocystis_sinensis_KT901190
Sarcocystis_bovifelis_KT901194
Sarcocystis_tenella_MF039319
Sarcocystis_cruzi_EF622176
Besnoitia besnoiti EU789637
Theileria-annulata AY684845
Cryptosporidum-parvum AF93009
Bos-taurus NR036644


210 220 230 240 250 260 270 280 290 300
Neospora-caninum L49389
Toxoplasma-gondii KX459518
Hammondia-hammondia DQ022686
Hammondia-heydorni GQ984218
Hammondia-triffittae GQ984215
Sarcocystis_buffalonis_KU24793
Sarcocystis_fusiformis_KR18615
Sarcocystis_buffalonis_KU24793
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Besnoitia besnoiti EU789637
Theileria-annulata AY684845
Cryptosporidum-parvum AF93009
Bos-taurus NR036644
310 320 330 340 350 360 370 380 390 400
Neospora-caninum L49389
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Sarcocystis_hirsuta_KT901232
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Sarcocystis_cruzi_EF622176
Besnoitia besnoiti EU789637
Theileria-annulata AY684845
Cryptosporidum-parvum AF93009
Bos-taurus NR036644

FIGURE 2. 6. SEQUENCE ALIGNMENT OF THE ITS1 GENE REGION OF PROTOZOAN SPECIES INCLUDED IN THIS STUDY GENERATED FROM THE OUTER PRIMER SET 'NTH-ITS1-F1 AND NTH-ITS1-R1': DASHES REPRESENT IDENTICAL BASE PAIR CHANGE, AND DOTS REPRESENT GAPS IN THE ALIGNMENTS. THE PRIMERS HIGHLIGHTED AMPLIFY: RED- *NEOSPORA*, *TOXOPLASMA* AND *H. HAMMONDIA*, BLUE- *NEOSPORA* AND GREEN- *TOXOPLASMA* AND *H. HAMMONDIA* BASED ON *IN SILICO* ANALYSIS.

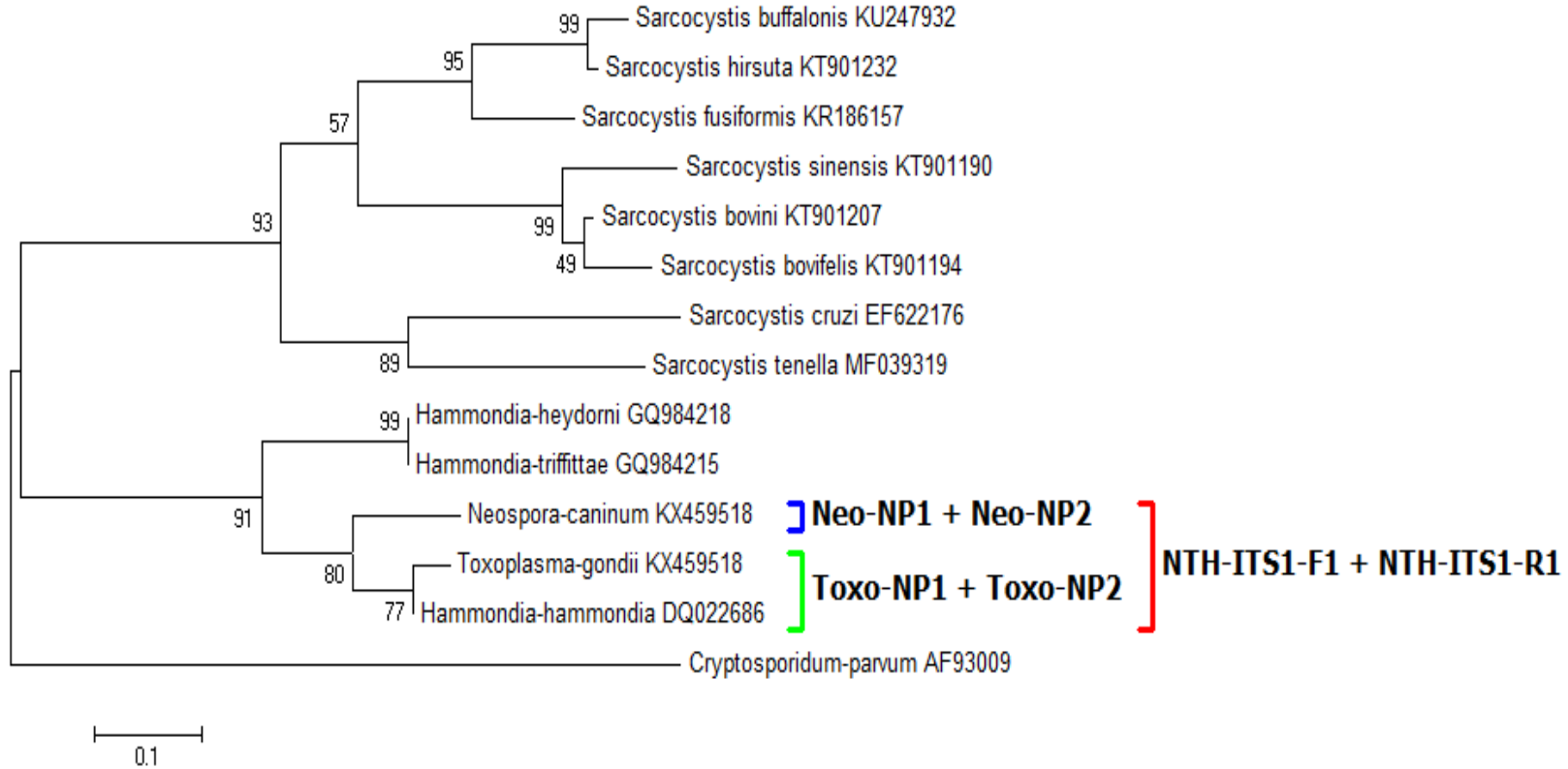


FIGURE 2. 7. ITS1 PHYLOGENETIC TREE SHOWING THE DIVERSITY AND COMPLEXITY OF THE PROTOZOAN SPECIES: *SARCOCYSTIS* SPP., *TOXOPLASMA* AND *NEOSPORA*. DEVELOPED PRIMERS ARE HIGHLIGHTED IN BRACKETS. THE PRIMERS HIGHLIGHTED AMPLIFY: *RED- NEOSPORA, TOXOPLASMA AND H. HAMMONDIA, BLUE- NEOSPORA AND GREEN- TOXOPLASMA AND H. HAMMONDIA BASED ON* IN SILICO.

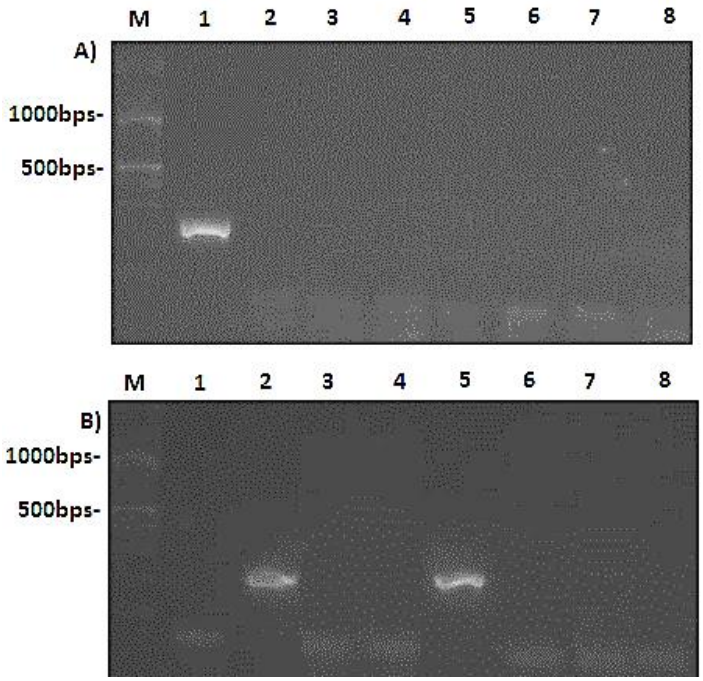


FIGURE 2. 8. SPECIFICITY PCR OF THE NEO-NP1 + NEO-NP2 AND TOXO-NP1 + TOXO-NP2 PRIMERS AT 55 °C. M REPRESENTS A 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- *NEOSPORA*, 2- *TOXOPLASMA*, 3- *S. TENELLA*, 4- *S. NEURONA*, 5- *H. HAMMONDIA*, 6- *H. HEYDORNI*, 7- *H. TRIFFITTAE* AND 8- DH$_2$O.

The previously published ITS1 inner primers were shown to work at an optimum temperature of 55º C. *Neospora* specific inner primers were shown to amplify only *Neospora* DNA, and *Toxoplasma* specific primers were shown to amplify *Toxoplasma*, and *H. hammondia* DNA (**Figure 2. 8**). No amplification with *H. triffittae*, *H. heydorni*, and *Sarcocystis* spp. DNA was observed (**Figure 2. 8**).

2.3.4. Identification and development of 18S *Sarcocystis* spp. group primers

The multi-sequence alignment in **Figure 2. 1** shows specific polymorphic sites that were used to develop various group primers. The phylogenetic analysis showed the diversity and complexity of the 18S region for the *Sarcocystis* spp. (**Figure 2. 4**). From **Figure 2. 4**, it can be seen that the 18S region shows a higher polymorphism for *Sarcocystis* spp. compared to *Neospora* and *Toxoplasma*. Three distinct clades for the *Sarcocystis* spp. were seen in the phylogenetic analysis (**Figure 2. 4**). Group primers were designed to amplify *Sarcocystis* spp. from each of the clades. Four forward

primers and four reverse primers that amplify various groups of *Sarcocystis* spp., were designed: **Table 2. 4.** Representative outer (general) and inner (specific) primers designed from the 18S rRNA gene and species that are most likely to be amplified based in *silico analysis* Results showed amplification at an optimum temperature of 58°C for each primer.

For each primer set, polymorphic sites of each primer at the 3' prime end for forward primers and 5' end for the reverse primers were observed. The polymorphic region on the primer binding site shows at least 2 base pair differences compared to the other species from different groups. Each group primer amplified a representative *Sarcocystis* species from each of the three clades shown from the phylogenetic tree (**Figure 2. 4**). Each *Sarcocystis* group primer can be used in combination with each other to give a greater specificity, amplifying different sets of species depending on the combination used. A **Table III** showing each primer combination and which species that will be amplified based on *in silico* analysis is shown in **Appendix I.**

2.3.5. Specificity of the *Sarcocystis* group primers

Results for group 1 primers (S-18S-G1 + NTS-18S-R2) revealed that the positive control *S. gigantean* DNA was amplified, whereas any other *Sarcocystis* species, *Neospora, Toxoplasma* and *Hammondia* DNA were not amplified (**Figure 2. 9**). Similar results were observed for the group 2 primer set (S-18S-G2 + NTS-18S-R2), which showed to be specific to *S. hirsuta* and *S. buffalonis,* whereas no other *Sarcocystis* spp., *Neospora, Toxoplasma* and *Hammondia* spp. DNA was amplified (**Figure 2. 10**).

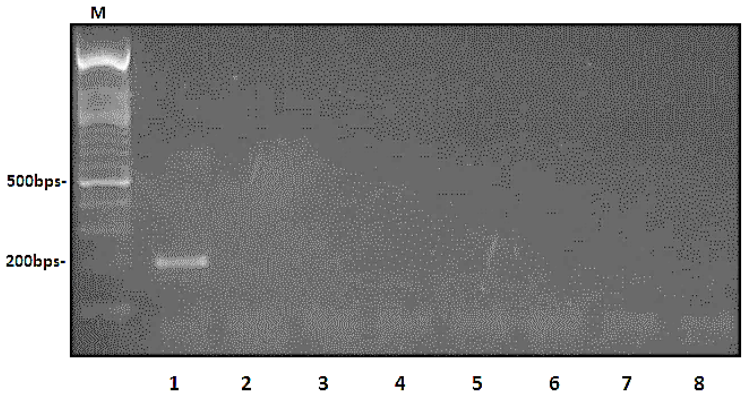


FIGURE 2. 9. PCR TO TEST THE SPECIFICITY OF GROUP 1 SPECIFIC PRIMER S-18S-G1 + NTS-18S-R2. M REPRESENTS A 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- *S. GIGANTEA*, 2- *S. FUSIFORMIS*, 3- *S. TENELLA*, 4- *S. FAYERI*, 5- *S. CRUZI*, 6- *S. ROMMELI*, 7- *S. BUFFANLONIS* AND 8- DH$_2$O.

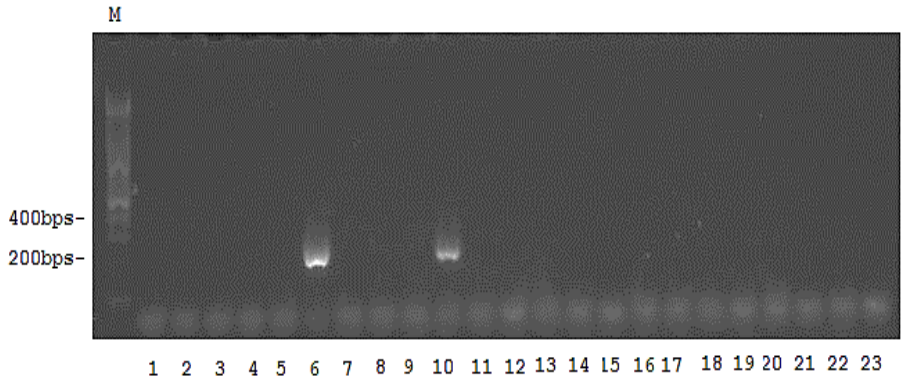


FIGURE 2. 10. PCR TO TEST THE SPECIFICITY OF GROUP 2 SPECIFIC PRIMER S-18S-G2 + NTS-18S-R2. M REPRESENTS A 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- *N. CANINUM*, 2- S. *FUSIFORMIS*, 3- *T. GONDII*, 4- *S. LEVINEI*, 5- *H. HAMMONDIA*, 6- *S. HIRSUTA*, 7- *H. HEYDORNI*, 8- *S. BOVIFELIS*, 9- *H. TRIFFIATTAE*, 10- *S. BUFFANLONIS*, 11- *S. LUTRAE*, 12- *S. SINENSIS*, 13- *S. TENELLA*, 14- *S. ROMMELI*, 15- *S. NEURONA*, 16- *S. CRUZI*, 17- *S. FAYERI*, 18- *S. HOMINIS*, 19- *S. RILEYI*, 20- *S. AUCHENIE*, 21- *S. BOVINI*, 22- *S. GIGANTEAN* AND 23- DH$_2$O.

Analysis of the group 3 primer set (S-18S-G3 + NTS-18S-R2) revealed that it would only amplify *S. bovifelis, S. bovini, S. sinensis, and S. rommeli* (**Figure 2. 11**). The group 4 primer set (S-18S-G5 + NTS-18S-F2) showed successful amplification of *S. fayeri, S. gigantean, S. bovifelis, S. hirsuta, S. bovini, S. buffalonis, S. sinensis, S. fusiformis, S. rommeli, and S. aucheniae* DNA. No amplification of other *Sarcocystis* species, *Neospora, Toxoplasma* and *Hammondia* DNA was observed (**Figure 2. 12**).

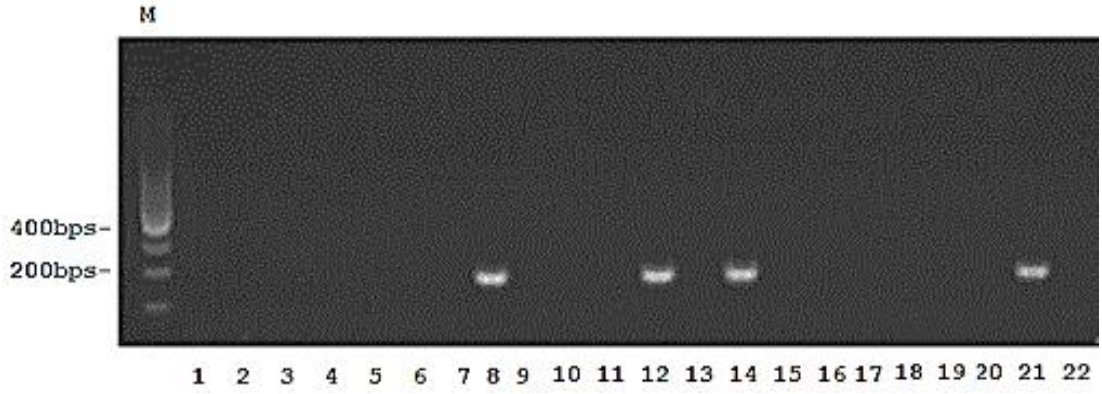


FIGURE 2. 11. PCR TO TEST THE SPECIFICITY OF GROUP 3 SPECIFIC PRIMER S-18S-G3 + NTS-18S-R2. M REPRESENTS A 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- *N. CANINUM*, 2- *S. FUSIFORMIS*, 3- *T. GONDII*, 4- *S. LEVINEI*, 5- *H. HAMMONDIA*, 6- *S. HIRSUTA*, 7- *H. HEYDORNI*, 8- S. *BOVIFELIS*, 9- *H. TRIFFIATTAE*, 10- *S. BUFFANLONIS*, 11- *S. LUTRAE*, 12- *S. SINENSIS*, 13- *S. TENELLA*, 14- *S. ROMMELI*, 15- *S. NEURONA*, 16- *S. CRUZI*, 17- *S. FAYERI*, 18- *S. HOMINIS*, 19- *S. RILEYI*, 20- *S. AUCHENIE*, 21- *S. BOVINI*, AND 22- dH_2O.

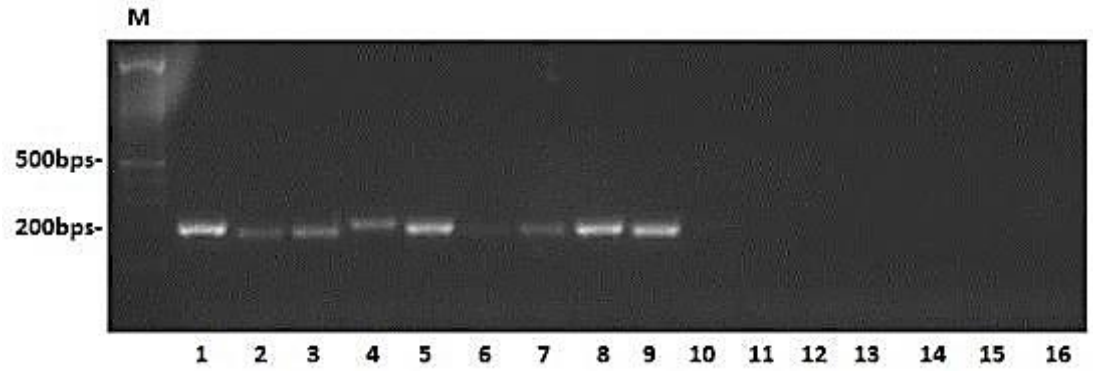


FIGURE 2. 12. PCR TO TEST THE SPECIFICITY OF GROUP 4 SPECIFIC PRIMER NTS-18S-F2 + S-18S-G4. M REPRESENTS A 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- *S. GIGANTEA*, 2- *S. SINENSIS*, 3- *S. FUSIFORMIS*, 4- *S. HIRSUTA*, 5- *S. BUFFANLONIS*, 6- *S. BOVINI*, 7- *S. ROMMELI*, 8- *S. AUCHENIAE*, 9- *S. BOVIFELIS*, 10- *S. FAYERI*, 11- *S. CRUZI*, 12- *S. TENELLA*, 13- *S. LEVENEI*, 14- *S. LUTRAE*, 15- *T. GONDII* AND 16-dH_2O.

Sarcocystis group 5 primer set (S-18S-G5 + NTS-18S-F2) only amplified *S. tenella, S. cruzi*, and *S. levinei* DNA (**Figure 2. 13**). No amplification of other *Sarcocystis* species, *Neospora, Toxoplasma* and *Hammondia* DNA, was observed. The group 6 primer set (S-18S-G6-F2 + S-18S-G6-R2) showed only amplification of *S. tenella* DNA and no amplification of any other *Sarcocystis* spp. *Neospora, Toxoplasma* and *Hammondia* spp. was observed (**Figure 2. 14**). The group 9 primer set (S-18S-G9 + NTS-18S-F2) showed to be specific and only amplified *S. neurona, S. lutrae* and *S. rileyi* DNA (**Figure 2. 15**).

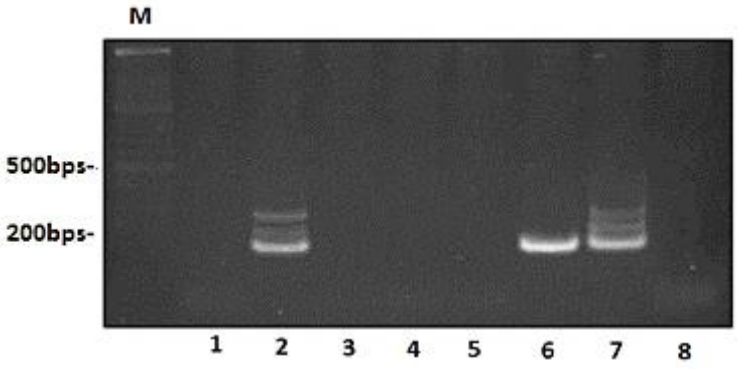


FIGURE 2. 13. PCR TO TEST THE SPECIFICITY OF GROUP 5 SPECIFIC PRIMER NTS-18S-F2 + S-18S-G5. M REPRESENTS A 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- *N. CANINUM*, 2- *S. CRUZI*, 3- *S. HIRSUTA*, 4- *S. BUFFALONIS*, 5- *S. BOVIFELIS*, 6- *S. LEVINEI*, 7- *S. TENELLA* AND 8- DH$_2$O.

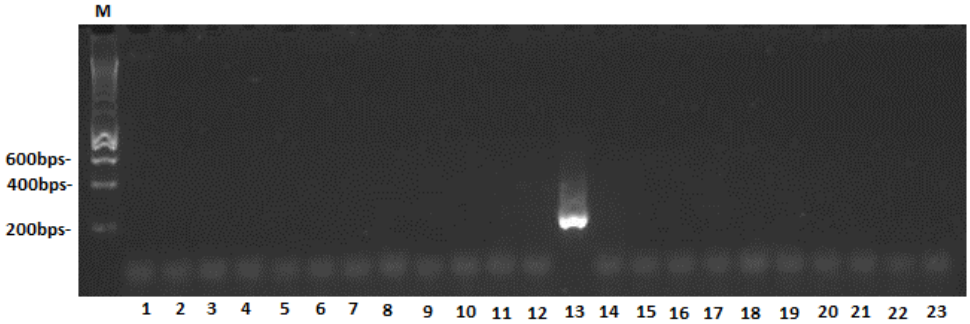


FIGURE 2. 14. PCR TO TEST THE SPECIFICITY OF GROUP 6 SPECIFIC PRIMER S-18S-G6-F + S-18S-G6 R. M REPRESENTS A 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- *N. CANINUM*, 2- *S. FUSIFORMIS*, 3- *T. GONDII*, 4- *S. LEVINEI*, 5- *H. HAMMONDIA*, 6- *S. HIRSUTA*, 7- *H. HEYDORNI*, 8- *S. BOVIFELIS*, 9- *H. TRIFFIATTAE*, 10- *S. BUFFANLONIS*, 11- *S. LUTRAE*, 12- *S. SINENSIS*, 13- *S. TENELLA*, 14- *S. ROMMELI*, 15- *S. NEURONA*, 16- *S. CRUZI*, 17- *S. FAYERI*, 18- *S. HOMINIS*, 19- *S. RILEYI*, 20- *S. AUCHENIE*, 21- *S. BOVINI*, 22- *S. GIGANTEA* AND 23- DH$_2$O.

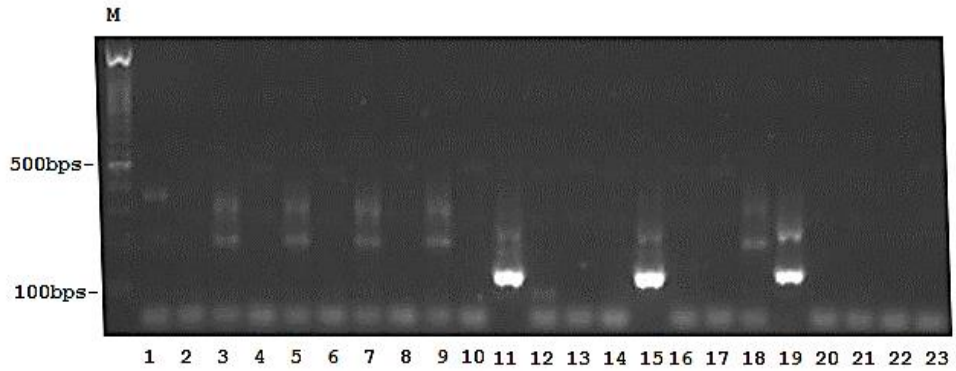


FIGURE 2. 15. NESTED PCR TO TEST THE SPECIFICITY OF GROUP 9 SPECIFIC PRIMER NTS-18S-F2 + S-18S-G9. M REPRESENTS A 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- *N. CANINUM*, 2- *S. FUSIFORMIS*, 3- *T. GONDII*, 4- *S. LEVINEI*, 5- *H. HAMMONDIA*, 6- *S. HIRSUTA*, 7- *H. HEYDORNI*, 8- *S. BOVIFELIS*, 9- *H. TRIFFIATTAE*, 10- *S. BUFFANLONIS*, 11- *S. LUTRAE*, 12- *S. SINENSIS*, 13- *S. TENELLA*, 14- *S. ROMMELI*, 15- *S. NEURONA*, 16- *S. CRUZI*, 17- *S. FAYERI*, 18- *S. HOMINIS*, 19- *S. RILEYI*, 20- *S. AUCHENIE*, 21- *S. BOVINI*, 22- *S. GIGANTEA* AND 23- DH$_2$O.

2.3.6. Validation of PCR primers

2.3.6.1. Validation of PCR primers using Moredun surveillance archived material from aborted ruminant cases

PCR primers were applied to aborted ruminants cases (61 cattle and 21 sheep) based on clinical/pathological, where protozoan aetiology were suspected to identify specific protozoan parasites, in order to validate the use of the PCR primers. All samples were tested using the 18S *Sarcocystis* group primers (NTS-18S-F2 + S-18S-G5-R, NTS-18S-F2 + S-18S-G4-R and S-18S-G6-F2 + S-18S-G6-R2) and the ITS1 *Neospora* and *Toxoplasma* specific primers (NT-ITS1-F + NT-ITS1-R, Neo NP1 + Neo NP2, and Toxo NP1 + Toxo NP2). Results showed that 32.1% (9/21, 95% CI:21.8– 66.1%) of ovine samples and 26.2% (16/61, 95% CI: 15.8– 39.1%) of bovine samples were positive for protozoan DNA using the pan protozoan primers NTS-18S-F1 + NTS-18S-R1. When comparing to previous IHC results using the Moredun In-house antisera 20 bovine samples were positive for *N. caninum* and 3 ovine samples were positive for *T. gondii*. No mixed infections were identified using the Moredun In-house antisera.

Successful amplification was observed for the above-mentioned primers, and sequencing results showed that 25.0% (7/21, 95% CI: 14.6 – 57.1%) and 10.7% (3/21, 95% CI: 3.0 - 36.3%) of ovine samples were positive for *Toxoplasma* and *S. tenella* DNA, respectively. No *Neospora* DNA was detected in any of the ovine samples, and only 1 samples showed mixed infections with *Toxoplasma* and *S. tenella* (**Appendix I Table IV A and B**). Moreover, sequencing results revealed that 19.7% (12/61, 95% CI: 10.6 – 31.8%), 4.9% (3/61, 95% CI: 1.0 - 13.7%) and 1.6% (1/61, 95% CI: 0.04 - 8.8%) of bovine samples were positive for *Neospora*, *Toxoplasma* and *S. cruzi* DNA, respectively. One bovine samples showed mixed infections with *Neospora*, and *S. cruzi* (**Appendix I Table IV A and B**).

Results showed successful amplification of protozoan DNA from fixed brain, heart and placenta samples from aborted foetuses, and revealed that 30.5% (25/82) (95% CI: 20.8 - 41.6%) of samples were positive for protozoan DNA (using NTS-18S-F1 + NTS-18S-R1) for both cattle (n= 61) and sheep (n= 21), whereas only 23 cases were

shown positive using the Moredun In-house antisera for protozoa (**Appendix I Table IV A and B**). In general results showed amplification of *Toxoplasma* DNA in 12.2% (10/82, 95% CI: 6.1 – 21.3%), and *Neospora* DNA in 14.6% (12/82, 95% CI: 7.8 – 24.2%) of ruminant samples tested using the 18S specific group primers (NTH-18S-F2 + NTH-18S-R2) and ITS1 specific primers (Neo NP1 + Neo NP2, and Toxo NP1 + Toxo NP2). Lastly, results showed that 4.9 (4/82, 95% CI: 1.3 – 12.0%) ruminant samples were positive for *Sarcocystis* DNA using the 18S *Sarcocystis* group primers.

2.3.6.2. Validation of PCR primers using badger samples

Badger samples from leg, neck muscle, tongue, sub-mandibular lymph node, liver, lung, brain, heart and spleen, were initially screened and tested using the 18S external primers (**Appendix I Table IV B**). Results revealed positive PCR amplicons in 1/5 leg muscle, brain and lung samples, 2/5 neck muscle samples and 3/5 tongue and spinal cord samples. Sequencing results revealed the identity to be *S. lutrae* (accession KM657770) in neck and tongue samples.

All available samples from the tongue (n= 32) and neck muscle (n= 54) from 54 badgers were tested using the *Sarcocystis* specific 18S nested PCR (NTS-18S-F1 and NTS-18S-R1, and S NTS-18S-F2 + S-18S-G9). Results revealed that 36/54 (67%) (95% CI: 52.5% - 78.9%) of neck samples and 24/32 (75%) (95% CI: 56.5% - 88.5%) tongue samples tested positive for *Sarcocystis* DNA. Twenty badger samples (20/32) (95% CI: 43.6% - 78.9%) showed positive PCR results for *Sarcocystis* DNA in both neck and tongue samples using the 18S PCR (**Appendix I Table IV B**). Forty badgers (40/54) (74%) (95% CI: 60.3% - 85.0%) tested positive for the *Sarcocystis* specific 18S PCR in at least one tissue. Sequencing results revealed that the *Sarcocystis* DNA amplified showed 100% identity to *S. lutrae*. Full study results are presented in Lepore *et al.* (2017).

2.3.6.3. Validation of PCR primers using suspected *Sarcocystis* cases from fresh and fixed tissue samples

Lastly, samples from various host muscle tissues (horse, capercaillie, pine marten, sheep, cattle, bat and whale) that were sent to Moredun pathology were tested using

the 18S PCR primers. Control material was used as per **Table 2.2**. All PCR positive samples were sent for sequencing, and the results revealed that various *Sarcocystis* species were amplified using the 18S PCR primers (**Appendix I Table IV A and B**). Results revealed that based on clincal presentations from aborted foetal tissue samples, that 3/5 ovine samples were positive for *T. gondii* DNA, 1/5 was positive for both *T. gondii* and *S. tenella* DNA and 2 ovine samples were negative for protozoan DNA. Three bovine samples (3/3) were positive for *N. caninum* DNA. Moreover, results revealed that muscle samples selected based on previous findings of cysts and lesions from previous histophatology revealved that 4/6 bovine samples were positive for *S. cruzi* DNA and 2 were negative. Results for ovine muscle samples revealed that 8/8 ovine samples were positive for protozoan DNA, of which 4 samples were positive for *S. tenella* DNA and 4 samples were positive for *S. gigantea* DNA (**Appendix I Table IV A and B**). A total of 14 horse, 2 cappercailles, 2 pine marten and 1 mink samples with histophatoloigical detections of cysts in the muscle tissue were tested by 18S PCR. Sequencing results revealed that 10 horse samples were positive for *S. fayeri* DNA, 2 cappercailles were positive for *S. rileyi* DNA and 2 pine marten and 1 mink sample were positive for *S. lutrae* DNA. One bat and one whale sample were tested, and results revealed that both samples were negative for protozoan DNA (**Appendix I Table IV A and B**).

2.3.6.4. Summary of validation of PCR primers

Extracted DNA samples from fresh (n= 81) and fixed (n= 98) tissues were screened using the 18S PCR assay for the diagnosis of protozoan species (**Appendix I Table IV A and B**). Host samples were tested with various PCR primers (**Table 2. 6**). Results revealed that protozoan DNA for *Toxoplasma*, *Neospora* and various *Sarcocystis* spp. were successfully amplified using the general PCR primers from ovine, bovine, horse, capercaillie, badger and pine marten (**Table 2. 6**). Successful differentiation of *Toxoplasma* and *Neospora* from *Sarcocystis* positive samples were observed from ovine, bovine, and badger tissues. Moreover, using the 18S specific *Sarcocystis* group primers enabled detection of *Sarcocystis* spp. from ovine, bovine, horse, capercaillie, badgers and pine marten samples (**Table 2. 6**). The ITS1 results revealed a genus-specific detection of *Neospora* and *Toxoplasma* DNA from ruminant samples (**Table 2. 6**).

TABLE 2. 6. SUMMARY OF HOST SAMPLES TESTED WITH THE DEVELOPED 18S AND ITS1 PCR PRIMERS

Loci	Primer set	Species detected	Samples applied to
18S	NTS-18S-F1 + NTS-18S-R1	*T. gondii, N. caninum, Hammondia* spp., *Sarcocystis* spp. *(Full list **Appendix Table III**)*	Ovine, Bovine, Horse, Capercaillies, Badgers, Pine Martens, Bat, Whale.
	NTS-18S-F2 + NTS-18S-R2	*T. gondii, N. caninum, Hammondia* spp., *Sarcocystis* spp. *(Full list **Appendix Table III**)*	Ovine, Bovine, Horse, Capercaillies, Badgers, Pine Martens, Bat, Whale.
	NTH-18S-F3 + NTH-18S-R3	*N. caninum, T. gondii, H. hammondia, H. heydorni, H. triffittae*	Ovine, Bovine, Horse, Capercaillies, Badgers, Pine Martens, Bat, Whale.
	NTS-18S-F2 + S-18S-G4	*S. silva, S. tarandi, S. sinensis, S. rangiferi, S. fusiformis, S. moulei, S. gigantea, S. scandinavica, S. oviformis, S. ovalis, S. fayeri, S. hardangeri, S. buffalonis, S. hirsuta, S. hominis and S. bovini*	Ovine, Bovine, Pine Martens and Badgers
	NTS-18S-F2 + S-18S-G5	*S. tenella, S. arieticanis, S. grueneri, S. alces, S. capreolicanis, S. gracilis, S. alceslatrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. heydorni and S. hircanis,*	Ovine, Bovine, Pine Martens and Badgers
	S-18S-G6-F + S-18S-G6-R	*S. tenella*	Ovine and Bovine.
	NTS-18S-F2 + S-18S-G9	*S. neurona, S. lutrae, S. lacerate, S. rileyi*	Capercaillies, Badgers, Pine martens.
ITS1	NTH-ITS1-F + NTH-ITS1-R	*N. caninum, T. gondii, H. hammondia*	Ovine, Bovine and Badgers.
	Neo NP1 + Neo NP2	*N. caninum*	Ovine, Bovine and Badgers.
	Toxo NP1 Toxo NP2	*T. gondii, H. hammondia*	Ovine, Bovine and Badgers.

Chapter 2.4. Discussion

Protozoal diseases causing reproductive disorders, such as abortions and stillbirth, are commonly associated with toxoplasmosis in sheep, and neosporosis in cattle, whilst eosinophilic myositis is most commonly associated with sarcocystosis in ruminants (Dubey *et al.*, 2015a; Dubey & Lindsay, 2006; More *et al.*, 2008). The diagnosis of *Sarcocystis* spp. can be accomplished by typical microscopic findings. However, techniques such as IHC and PCR are necessary for the identification of speccific protozoan parasites in affected tissue to establish a aetiological diagnosis (Dubey & Schares, 2006; More *et al.*, 2008).

Nowadays, molecular diagnostics are more frequently used for the detection of *Neospora*, *Toxoplasma* and *Sarcocystis* spp. However, these protozoan parasites share many common characteristics and show a close homology at the molecular level. Hence, distinguishing between these related protozoans has proven difficult. Conventional PCR can play an important role in the diagnosis of abortion cases using tissues samples, such as brain, placenta and muscle from aborted foetuses. However, whilst conventional PCR might be specific for the targeted organism, sensitivity may be relatively low. Due to low numbers of parasites present in tissues or degradation of clinical samples from processes, such as prolonged fixation or ageing, amplification might be problematic (Dubey *et al.*, 1988a; Gilbert *et al.*, 2007; Hurtado *et al.*, 2001; Lin *et al.*, 2009). The nested PCR has been shown to have a higher sensitivity compared to a single round PCR, especially from suboptimal nucleic acid samples (such as those extracted from formalin-fixed and paraffin-wax-embedded tissues) (Lin *et al.*, 2012). The PCR method in this study uses the initial PCR reactions as a template for the second round of amplification, and only if the first round PCR product was amplified will the second reaction generate a product of the desired size (Wilczynski, 2009). However, even though nested PCR increases sensitivity, false positive PCR products may occur from amplification of non-specific sequences and from contamination. Problems with this technique should hence always be considered.

PCR primers amplifying smaller fragments of under 500 base pairs were developed, as amplification of smaller amplicons has previously been shown to be successful,

especially from fixed tissue samples (Gilbert *et al.*, 2007; Hurtado *et al.*, 2001; More *et al.*, 2008). This chapter discusses the production of a nested PCR that detects and distinguishes DNA and thus potential infections of *Neospora*, *Toxoplasma* and various *Sarcocystis* spp. from fresh and fixed tissue samples. Two target regions, ITS1 region and 18S rRNA gene, were chosen for primer design for nested PCR primers. Firstly, the 18S rRNA gene was used due to its highly conserved regions and repetitive nature among related apicomplexan parasites. There was also extensive information available on the 18S rRNA gene, especially for the *Sarcocystis* genus and *Neospora, Toxoplasma* and *Hammondia* spp., (at the time of primer design) compared to the ITS1 region (Yang *et al.*, 2001b). Five external primers were developed and used as a universal primer to enable amplification of apicomplexan DNA (*Neospora, Toxoplasma*, *Sarcocystis* spp. and *Hammondia* spp.), yet exclude other related protozoan DNA (*C. parvum, Babesia, Theileria*), as well as host species (*bovine/ ovine/ Homo sapien*). The general PCR primers were developed from a more conserved region shown in the multi-sequence alignment. Various studies have demonstrated that the genomic sequences of DNA coding for the 18S rRNA gene and ITS1 region can be a good marker for phylogenetic studies, and promising targets for the development of species-specific PCR (Dubey & Schares, 2006; Ellis, 1998; Fischer & Odening, 1998; More *et al.*, 2008; Neefs *et al.*, 1991).

The hypervariability of the 18S rRNA gene has proven useful for the characterisation and identification of different species. However, due to the vast polymorphic sites among various *Sarcocystis* spp., in the 18S region, no single genus-specific primer was able to be designed. However, several *Sarcocystis* group primers were developed. The phylogenetic analysis has demonstrated a high diversity and complexity of the 18S rRNA gene for *Sarcocystis* spp., and distinct clades among the *Sarcocystis* spp. were seen. The *Sarcocystis* group primers were developed to detect the species that infect different intermediate hosts (sheep, cattle, goats and buffalo). From this study, primers S-18S-G2, S-18S-G3 and S-18S-G4 were mainly used in the diagnosis of *Sarcocystis* spp. in cattle and buffaloes, whereas the group primers S-18S-G1, S-18S-G5 and S-18S-G6 were mainly used in the diagnosis of *Sarcocystis* spp. in sheep and goats. The PCR primers NTS-18S-F2, NTS-18S-F2 and S-18S-G9 were able to amplify a range of *Sarcocystis* spp. found in other hosts, such as horses, carnivores, birds and other

mammalian species (Lepore *et al.*, 2017). A higher polymorphism of the genus *Sarcocystis* compared to *Neospora, Toxoplasma* and *Hammondia* spp. observed from the multi-sequence alignment and phylogenetic tree showed that the 18S primers developed were able to distinguish *Sarcocystis* spp. from *Neospora* and *Toxoplasma*. Moreover, the 18S rRNA gene has also been previously used in other studies for the identification of bovine *Sarcocystis* species (Fischer & Odening, 1998; Gjerde, 2016a; Vangeel *et al.*, 2013; Yang *et al.*, 2001b).

From the multi-sequence alignment and phylogenetic tree, it was however observed that the 18S rRNA gene showed limited polymorphism, particularly among very closely related groups. Similar results were shown in the study by Gjerde (2016a), who showed that even though sequence variation at the 5' end of the 18S rRNA gene was seen, an overlap between the intra- and inter- species-specific sequence divergence was observed. No obvious difference between *S. hirsuta* and *S. buffalonis* and *S. sinensis* from *S. bovifelis* and *S. bovini* were observed (Gjerde, 2016a). Hence, it was not possible to reliably distinguish between these five very closely related species based on the 18S rRNA gene sequences alone (Gjerde, 2016a; Yang *et al.*, 2001a). From the 18S phylogenetic tree and sequence alignment generated in this study, similar results between *S. gigantean* and *S. moulei* with a 98.5% identity, *S. cruzi* and *S. levinei* with 99.0% identity, and *S. sinensis* from *S. bovifelis* and *S. bovini* with 99.6% - 99.2% identity were observed. In previous studies by Gjerde (2016a) and Gjerde *et al.* (2015), only minor differences between very closely related *Sarcocystis* spp. in cattle and water buffalo were also seen in the 18S rRNA gene.

Additionally, only a few base pair differences between the related species *Toxoplasma, Neospora,* and *Hammondia* spp. were observed. From the 18S rRNA gene, a 99.4 – 99.6% identity was observed between *Neospora, Toxoplasma* and *Hammondia* spp., explaining why the 18S primers developed in this study showed cross-amplification of DNA from *Neospora* and *Toxoplasma*. Other studies have also shown that these minor differences found between the 18S rRNA gene of *T. gondii* and *N. caninum* could lead to difficulties for the development of species-specific primers (Ellis *et al.*, 1994; Holmdahl *et al.*, 1994; Marsh *et al.*, 1995). Our result showed a lack of polymorphic regions in the 18S rRNA gene between *Neospora, Toxoplasma* and *Hammondia* spp.

This meant that it was not possible to develop genus-specific primers able to distinguish between the closely related *Neospora* and *Toxoplasma* using the 18S rRNA gene, as primers were proven to be non-specific. On the other hand, general *Neospora* / *Toxoplasma* / *Hammondia* primers (NTH-18S-F2 and NTH-18S-R2) were developed. The general *Neospora* / *Toxoplasma* / *Hammondia* primers have proven to be specific, and able to distinguish *Neospora* / *Toxoplasma* / *Hammondia* from *Sarcocystis* spp., as enough polymorphism from *Sarcocystis* spp. was observed.

Using a conserved region with limited polymorphism amongst very closely related species can however lead to misidentification, especially if mutations during PCR or sequence analysis errors occur. As such, the ITS1 region was chosen as an additional locus to distinguish between *Neospora* from *Toxoplasma* and *Hammondia* spp. Using multiple loci, such as the 18S rRNA gene and the ITS1 region will help species identification and detection of protozoan DNA, as it is more reliable than using one locus alone. Similar results were also observed by Gjerde and Josefsen (2015) and Lepore *et al.* (2017), who showed that only a few polymorphic regions in the 18S rDNA were observed in very related *Sarcocystis* spp. Using an additional locus such as ITS1 can confirm the presence of protozoan DNA in the host. A higher polymorphism was observed from the ITS1 multi-sequence alignment between *Neospora* and *Toxoplasma*. The ITS1 phylogenetic tree demonstrated a high diversity level compared to the 18S rDNA among related species. Similar results shown by various studies have demonstrated that the ITS1 region gives a clearer differentiation for closely related species, such as *Neospora*, *Toxoplasma* and *Sarcocystis* spp. (Dubey & Schares, 2006; Ellis, 1998; Ellis *et al.*, 1999; Gjerde & Josefsen, 2015). Since the ITS1 region is not a gene, higher mutation densities are tolerated, making this region highly variable amongst species, and thus a useful marker for species identification. Many studies have therefore demonstrated and published protocols using the ITS1 region in molecular analysis. The numerous sequence differences and highly polymorphic ITS1 region allows the establishment of species-specific PCR amongst *T. gondii*, *N. caninum*, *H. heydorni* and *H. Hammondia* (Buxton *et al.*, 1998; Dubey & Schares, 2006; Ellis, 1998; Hurtado *et al.*, 2001; Payne & Ellis, 1996). The ITS1 region is an ideal candidate for PCR development because they are present at high copy number, exhibit interspecific variability and yet are highly conserved within a species

(Ellis *et al.*, 1999). The ITS1 region is easily isolated by PCR, due to its location between conserved rRNA genes (18S and 28S) and hence sequences have been fully characterised for *Neospora* and *Toxoplasma* (Homan *et al.*, 1997; Payne & Ellis, 1996). In this study a lack of conserved regions was seen from the ITS1 multi-sequence alignments among the protozoan *Neospora, Toxoplasma* and *Sarcocystis* spp., and hence no universal ITS1 primers were developed that enabled amplification of *Sarcocystis*, *Neospora* and *Toxoplasma* DNA. However, universal outer ITS1 primers that amplify *Neospora*, *Toxoplasma* and *H. Hammondia* were developed from this region. These outer primers enabled amplification of a smaller base pair fragment from *Neospora* / *Toxoplasma* / *H. hammondia* DNA, yet no amplification of *Sarcocystis* spp., other closely related protozoan (*Cryptosporidium, Theileria, Besnoitia*) or host DNA (bovine) was observed.

Previously, published ITS1 inner primers have been shown to amplify *N. caninum* (Buxton *et al.*, 1998) and *T. gondii* (Hurtado *et al.*, 2001). The *Neospora* PCR primers were shown to be specific and were optimal to be used with the external outer ITS1 primers in this study for diagnostics in ruminants, as they showed no amplification of *Toxoplasma* DNA. Similar results were seen for the ITS1 *Toxoplasma*-specific primers, which enabled amplification of only *T. gondii* DNA, as no cross-amplification of *Neospora* was observed. The ITS1 region for *Toxoplasma* and *Neospora* has been shown to vary in length between 392 and 421 base pairs respectively, and had shown a homology of 82.9% (Holmdahl & Mattsson, 1996; Hurtado *et al.*, 2001). A 23 base pair insertion was observed in the ITS1 region of *N. caninum*, making this region a good candidate for a species-specific primer design (Hurtado *et al.*, 2001). However, from the multi-sequence analysis, a low polymorphism was observed between the closely related species *T. gondii* and *H. hammondia*, and it was shown that primers also amplified *H. hammondia*. In the study by Hurtado *et al.*, (2001), the internal primer pair was designed to bind to a highly divergent region to ensure the specificity of the assay, yet it was predicted that the closely related species *H. hammondia* might also be amplified (Hurtado *et al.*, 2001). *Hammondia hammondia* is known to be a close but avirulent relative of *T. gondii*, and only a few base pair differences were observed in the ITS1 sequence alignment making differentiation challenging (Walzer *et al.*, 2013). However, sheep have not been previously described as an intermediate

host for *Hammondia,* and hence amplification of this species in ruminant samples can be eliminated (Hurtado *et al.*, 2001). Using the *Neospora* and *Toxoplasma*-specific inner primers enables the amplification of *Neospora* and *Toxoplasma* DNA from various ruminant species without the risk of cross-amplification. Even though *N. caninum* is predominantly associated with bovine tissue, and *T. gondii* is mainly associated with ovine tissue, using the ITS1 specific PCR primers enables detection of *Neospora* DNA in sheep and *Toxoplasma* DNA in cattle.

Initially, only limited *Sarcocystis* spp. sequences information previously identified in ruminants was available for the ITS1 primer development, and hence no *Sarcocystis* genus-specific primers were developed. More recently, ITS1 sequences of *Sarcocystis* spp. found in ruminants (especially cattle) have become available, and were included in the phylogenetic analysis and multi-sequence alignment as a comparison to the 18S rRNA gene (Gjerde, 2016a; Gjerde & Josefsen, 2015; Yang *et al.*, 2001b). The ITS1 alignment showed a higher polymorphism within the *Sarcocystis* genus compared to *Neospora* and *Toxoplasma*. However, genus-specific primer development based on conserved regions was not possible, as there were too many polymorphic regions among the genus *Sarcocystis*. It was not possible to design primers to amplify small fragments of less than 500 base pairs to enable genus-specific amplification. Several studies have used the ITS1 region to identify *Sarcocystis* spp. within avian and carnivore intermediate hosts, since this region has shown a higher level of sequence divergence between related species compared to the 18S gene (Gjerde, 2014; Gjerde & Josefsen, 2015). Differences between the ITS1 sequences of several cattle *Sarcocystis* spp. were observed due to varying length and substitutions within the sequence (Gjerde, 2016a). More sequence divergence compared to the 18S rRNA gene was observed between very closely related species, i.e. *S. buffalonis* and *S. hirsuta* (82.6% - 94.4%) and *S. sinensis*, *S. bovifelis* and *S. bovini* (83.4% – 87.0%). More recently, it was suggested that using the mitochondrial Cytochrome c oxidase subunit I gene (cox1) gene as an additional marker choice for the identification of closely related *Sarcocystis* spp. would be superior to the 18S rRNA gene and ITS1 region in the ability to distinguish between closely related *Sarcocystis* spp. in ruminants (Gjerde, 2016a). Only recently have the COX1 gene sequences of *Sarcocystis* spp. in ruminants become available and could be used for primer development in future diagnostics.

Difficulties have been described in other studies, reporting considerable differences in sequences in closely related species, as sequence variation between gene copies within a single *Sarcocystis* species, strain variation or even mixed infections with multiple species are possible (Gjerde, 2016a; Vangeel *et al.*, 2013). Mixed infections with *Neospora*, *Toxoplasma* and *Sarcocystis* spp. are often seen, however, they are not frequently reported (Gjerde & Josefsen, 2015; Katzer *et al.*, 2014; Lepore *et al.*, 2017; More *et al.*, 2008; Unzaga *et al.*, 2014). Many studies tend to specifically look for only one protozoan species and hence miss infections with multiple species. Ruminants can often serve as intermediate hosts to multiple infections, and therefore most hosts are often infected by several different protozoan species (Gjerde, 2016a; More *et al.*, 2008; Yang *et al.*, 2001b). In the study by Katzer *et al.*, (2014), where mixed infections of *S. tenella* in *T. gondii* infected sheep were observed in the heart and muscle tissues from all animals studied. Moreover, in the study by More *et al.*, (2008), coinfection of *S. cruzi* with *N. caninum* and *T. gondii* has been reported by indirect fluorescent antibody testing in Argentinean beef cattle. *Sarcocystis tenella* and *S. cruzi* are known as pathogenic species, and sarcocystosis in *T. gondii* infected sheep, and in *N. caninum* infected cattle have been reported more frequently.

Using a species-specific primer alone might hinder detection of mixed infections, which are nowadays more frequently reported in ruminants. However, using solely universal protozoan primers might hinder correct sequence diagnosis, as multiple sequences may be observed in sequence analysis making identification of a single species difficult. Mixed infections can be identified using the combination of nested PCRs developed here, as the 18S PCR primers can eliminate and detect multiple infections with *Toxoplasma, Neospora* and *Sarcocystis* spp. in ruminants and other animal host species. Our study describes the detection of mixed infection of *Toxoplasma* and *S. tenella* in an ovine sample and *Neospora* and *S. cruzi* in a bovine sample. The 18S *Sarcocystis* group primer only amplifies *Sarcocystis* species, and the 18S NTH primer only amplifies *Neospora* / *Toxoplasma* and *Hammondia* spp. When both PCRs are shown positive, it can be assumed that mixed infections are present. Samples can be more easily confirmed by sequencing for the presence of the protozoal species. Mixed infections could not be detected by using the Moredun In-house

antisera alone and one can argue that the PCR assay may hence be more sensitive in comparison to using polyclonal antisera.

The PCR primers designed in this study were validated and applied to several host species (ruminants, wild carnivore, horse, bat, bird and whale samples). Results showed amplification of various *Sarcocystis* species including *S. lutrae*, *S. fayeri*, *S. rileyi, S. cruzi, S. gigantean* and *S. tenella*, as well as *Neospora* and *Toxoplasma* using the 18S and ITS1 PCR primers. Each species was confirmed by sequencing of the PCR products. These PCR primers were able to amplify protozoan species from both fixed and fresh tissue samples, suggesting that amplification using the 18S rRNA gene and the ITS1 region from various host species is possible. In this study, 82 ruminant samples were selected to identify specific protozoan parasites in abortion cases where protozoal aetiology were suspected. The successful amplification of protozoan DNA (*T. gondii, N. caninum, S. cruzi* and *S. tenella*) from fixed tissue samples suggest that these PCR primers could be used to identify to presence of protozoan parasites DNA in ruminant abortion cases. In our study two samples had shown mixed infections with *N. caninum* and *S. cruzi,* and *T. gondii* and *S. tenella,* showing that this PCR was able to detect mixed species of protozoan.

Moreover, the 18S *Sarcocystis* group primers used on the badger's samples showed that the primers were able to successfully differentiate *Sarcocystis* DNA from *Toxoplasma* and *Neospora* in the same carnivore host and enable accurate identification of the protozoan DNA. Sequencing results confirmed that the DNA was shown to be 100% identical to *S. lutrae* (Lepore *et al.*, 2017). The same badger samples were previously tested were shown to be positive for *Toxoplasma* (25%) (Burrells *et al.*, 2013) and *Neospora* (10.9%) (Bartley *et al.*, 2013). The 18S PCR enabled detection of *Sarcocystis* spp., from host species harbouring DNA from mixed infections of *Toxoplasma* and *Neospora*. However, the 18S group primers were able to differentiate *S. lutrae* from *T. gondii* and *N. caninum*, suggesting that these 18S primers could be used in the differential diagnosis of different protozoan species within the same sample. The usefulness and validation of the PCR primers were additionally demonstrated through the amplification of *Sarcocystis* DNA from host species other than ruminant species (horse, pine marten, and capercaillies) with suspected protozoal

infections from both fixed and fresh tissue samples. Results showed that the PCR managed to amplify DNA from various *Sarcocystis* species, *S. lutrae*, *S. rileyi*, *S. gigantean*, *S. tenella*, *S. fayeri* and *S. cruzi*, from a wide range of host species. These results indicate that the PCR method developed could be applied to a wide range of host species, and enable the detection of DNA of protozoan species using a range of 18S and ITS1 primers. The 18S PCR primers enable a good detection at the genus level. However, using the 18S primers alone: it will not be possible to differentiate between *Neospora*, *Toxoplasma* and very closely related *Sarcocystis* species, due to their high homology within the 18S rRNA gene. The ITS1 region was used as an additional region to enable differentiation of DNA between closely related species, as it showed a higher polymorphism compared to the 18S rRNA gene. Specific PCR primers, such as the ones targeting the ITS1 region can enable species-specific diagnosis of *Sarcocystis* spp. DNA. However, at the time of the primer design, sequences for *Sarcocystis* spp. found in ruminants were unavailable, and hence no species-specific primers were designed. It has been advised to use the ITS1 region or the COX 1 gene for the diagnosis of protozoan due to their high polymorphism among closely related species (Gjerde, 2016a; Gjerde *et al.*, 2015).

As a conclusion, using molecular analyses, such as this nested PCR assay, has proven to be a useful diagnostic tool to detect and differentiate protozoan DNA from fixed and frozen tissue samples. The PCR assay may have an enhanced sensitivity due to the fact that it enables identification of mixed infections identified and a higher proportion of suspected protozoal species were identified in the samples. Yet, further evalution and testing of sensitivity between the two tests (PCR and IHC) are needed to confirm this. Additionally, PCR amplification only indicate the presence of parasite nucleic acids and further evaluation is needed to determine the presence and the cause of abortion from particular protozoan parasites. Hence this molecular diagnostic assay should be used in conjunction with additional diagnostic tests, such as histopathology and immunohistochemistry. Only tests, such as histopathology and IHC can verify whether the parasites detected are the causal agent, and the signs of an active infection with inflammatory lesions in the tissue and therefore the cause of abortion (Dagleish *et al.*, 2010; Pereira-Bueno *et al.*, 2003)

Chapter 3.

Chapter 3.1. Improved IHC diagnosis – genus-specific recombinant protein production

3.1.1. Introduction

As stated in Chapter 1, the tissue-dwelling coccidian parasites *N. caninum, T. gondii* and *Sarcocystis* spp. share many similarities. Each parasite has been shown to develop in intermediate hosts, move between definitive and intermediate hosts, and reproduce asexually and sexually (Dubey & Lindsay, 2006; Reid *et al.*, 2012). *Toxoplasma* is known to complete the sexual stages of its life cycle in felids, whilst, *Neospora* undergoes the sexual stages exclusively in canids (McAllister *et al.*, 1998). *Sarcocystis* spp. are known to have a variety of definitive hosts, and various *Sarcocystis* species have been identified in ruminants (Dubey *et al.*, 2015a; Dubey & Lindsay, 2006; Dubey *et al.*, 1989b; Kaltungo & Musa, 2013). *Neospora* and *Toxoplasma* share a high degree of homologous antigens, and thus share similar conformational structures (Howe & Sibley, 1999). This high degree of homology can potentially lead to cross-reactivity in techniques used to distinguish between the different protozoans. Cross-reactive antigens have previously been identified, and antibodies have been shown to react with other morphological similar cyst-forming parasites, i.e. between *Neospora, Toxoplasma* and *Sarcocystis* (Gondim *et al.*, 2017; Liao *et al.*, 2005; McAllister *et al.*, 1996b). Cross-reactivity between *Neospora* and *Toxoplasma* has been observed when using whole *Neospora* tachyzoite lysates during enzyme-linked immunosorbent assays (ELISA) (Chahan *et al.*, 2003). Cross-reactive antigens make diagnosis and differentiation particularly difficult in abortion cases of ruminants, between these closely related protozoan.

Each protozoan parasite differs in the molecular determinant of host specificity and varies at the molecular level of genes and proteins (Reid *et al.*, 2012). Molecular determinants of host specificity, especially antigens that play a significant role in the

host cell invasion machinery, are likely to have a unique biological feature of each apicomplexan parasite (Reid *et al.*, 2012). The surface of each coccidian parasite is coated with highly abundant GPI-linked surface proteins known as surface antigens (SAG) and SAG-related sequences (SRS) (Boothroyd *et al.*, 1998; Sohn *et al.*, 2011). Genetic differences in the highly abundant family of genes, such as the SAG gene family, have been uniquely identified between *Neospora*, *Toxoplasma* and *Sarcocystis* spp. The *Toxoplasma* antigens, such as TgSAG1 (P30), TgSAG3 (P41) and TgSAG2A (P22) and TgSRS2 (P43), are essential factors for parasite invasion of host cells that include cell attachment, interaction and survival in the host (Burg *et al.*, 1988; Cesbron-Delauw, 1995; Gondim *et al.*, 2017; Prince *et al.*, 1990; Reid *et al.*, 2012). A more extensive repertoire of similar homologues in *Neospora*, i.e. NcSAG1 (P29), NcSAG2 (P22) and NcSRS2 (P43) was identified. However, these show considerable differences compared to *T. gondii* (Reid *et al.*, 2012).

Sarcocystis spp. are also known to exhibit levels of similarity between surface antigens. As of yet, only *S. neurona* SAG orthologues (i.e. SnSAG1, SnSAG2, SnSAG3, SnSAG4 and surface proteins SnSPR1) have been identified, and have been shown to be expressed during the invasion and evasion stages (Ellison *et al.*, 2002; Howe *et al.*, 2008; Howe *et al.*, 2005). Similar to *Neospora* and *Toxoplasma*, surface antigen SnSAG assists in virulence factors that are needed to invade cells and evade the host's immune response (Howe *et al.*, 2005; Yeargan & Howe, 2011). However, only limited information is available for genes on other *Sarcocystis* spp. such as *S. tenella*, and thus no comparisons can be made. Despite the largely conserved gene families among these protozoal species, numerous differences in gene expression especially in the SAG and SRS repertoire have been found, and could be used for the development of genus-specific recombinant proteins (Gondim *et al.*, 2017; Reid *et al.*, 2012).

These genetic differences in the parasite genome could be used to minimise the potential of cross-reactivity during pathological analysis such as IHC, due to their genus-specific nature. Various studies have shown that using recombinant proteins to develop diagnostic techniques can reduce this risk of cross-reactivity with other coccidia (Dong *et al.*, 2012; Gondim *et al.*, 2017; Jin *et al.*, 2015; Nishikawa *et al.*,

2001; Uzeda *et al.*, 2013). For example in the study by Gondim *et al.*, 2017, identified species-specific antigens and stage-specific proteins that have shown promising use for diagnosis using serological analysis (IFAT, ELISA and western blots). In the study by Jin *et al.*, 2015, who expressed a truncated dense granule protein 2 (NcGRA2t) showed that this protein was shown to be specific for *Neospora* in the serodiagnosis by ELISA in dogs. Using recombinant proteins has greatly expanded diagnostics, and resulted in the production of more sensitive and specific tools for the detections of protozoa such as *Toxoplasma* and *Neospora,* compared to using native antigens (Dong *et al.*, 2012; Gondim *et al.*, 2017; Kotresha & Noordin, 2010). *Escherichia coli* is known to be one of the most popular organisms for the expression of recombinant proteins due to its simplicity, and is the standard application in the production of clones of specific-epitopes used for the production of antibodies (De Marco, 2015).

Recombinant proteins produced in *E. coli* prokaryotic expression systems have been shown to be more specific and sensitive as no cross-reactivity between *Toxoplasma* and *Neospora* was observed and as the percent positive value was higher using recombinant protein when compared to commercial ELISA kits where whole parasite lysates were used (Dong *et al.*, 2012; Kotresha & Noordin, 2010). Moreover, using bacterial systems to express specific recombinant proteins could be used to limit the cross-reactive potential with homologue proteins from closely related species. Production of recombinant proteins in bacterial expression systems has been shown to be a more cost-effective method of producing large quantities of high-quality proteins that can be used for genus-specific antibody development (Pietkiewicz *et al.*, 2004). Expression of recombinant proteins of *T. gondii*, *N. caninum* and *Sarcocystis spp*. are more frequently being used to develop specific diagnostic tools, such as immunoassays (Dong *et al.*, 2012; He *et al.*, 2013; Kotresha & Noordin, 2010; Uzeda *et al.*, 2013; Yeargan & Howe, 2011). However, despite the advantages of recombinant proteins, only a few diagnostic tools are available for the detection of *Neospora, Toxoplasma* and *Sarcocystis* spp. using IHC diagnostic techniques due to the difficulty of finding suitable expression system of toxic antigens and difficulty of purification of insolule recombinant proteins.

3.1.2. Aims and objectives

In this chapter genus-specific recombinant proteins will be developed to be used for the development of genus-specific antibodies. This will be achieved by:

- Identification of suitable target genes for the production of genus-specific recombinant proteins for *Neospora*, *Toxoplasma* and *Sarcocystis* spp.
- Expression of recombinant proteins using *E. coli* expression systems.
- Analysis of the developed recombinant proteins for cross-reactivity using immune serum that is positive for *Neospora*, *Toxoplasma* and *Sarcocystis* spp.

Chapter 3.2. Materials and Methods

3.2.1. Target genes of interest for recombinant protein production

Suitable target regions were identified using literature reviews and genome comparisons using *Toxoplasma*-database (ToxoDB) (http://www.toxodb.org) and NCBI (http://www.ncbi.nlm.nih.gov). Surface antigens and surface antigen-related genes were chosen for recombinant protein production based on the expression at different lifecycle stages (i.e. tachyzoites, bradyzoites or merozoites) and their potential for maximum labelling of the parasites. Target genes SAG, SRS and one organelle species-specific genes GRA were chosen as target genes for *Neospora* (Nc) and *Toxoplasma* (Tg) (**Table 3. 1**). Target genes for *S. tenella* (St) were initially investigated since this is a common pathogenic species in sheep in the UK. However, a *S. neurona* (SnSAG) and the surface protein (SnSPR1) gene were chosen for recombinant protein production due to the lack of genetic information available for the *S. tenella* genome (**Table 3. 1**). The *S. neurona (Sn*) genes were chosen as they are closely related to *S. tenella*, and that antibodies raised against the recombinant Sn proteins may cross-react against St proteins.

TABLE 3. 1. TARGET GENES FOR RECOMBINANT PROTEIN PRODUCTION FOR *NEOSPORA*, *TOXOPLASMA* AND *SARCOCYSTIS NEURONA*

Accession number (Database)	Protozoan parasite	Name	Product	Primer with Restriction sites (5'-3')
TGME49_233460 (ToxoDB)	*Toxoplasma gondii*	TgSAG1	SRS29B	gca tgc CTC GTG TGC GGG AAA GAT GGA aag ctt TCA CGC GAC ACA AGC TGC GAT A
TGME49_271050 (ToxoDB)		TgSAG2	SRS34A	gca tgc TTC AAG TTC GCT CTT GCG TCC A aag ctt ACG GCC ATC GGC ACC CGG A
TGME49_308020 (ToxoDB)		TgSAG3	SRS57	gca tgc AGG CAG TCT TTG CCG CTC GGG T aag ctt TCG CTG CTT GGC GGG CGG AAG G
TGME49_233480 (ToxoDB)		TgSRS2	SRS29C	gca tgc GTT GGT TGC AGG CAC AAC AC ctg cag AGA ATC GGA TCC TGC CAA ACC
NCLIV_021640 (Ncbi)	*Neospora caninum*	NcGRA7	GRA7	gga tcc GGC TGG AGA CTT GGC AAC CGA A ccc ggg CTA TTC GGT GTC TAC TTC CTG CTC
NCLIV_033250 (Ncbi)		NcSRS2	SRS2	ggt acc GGT GTC GGG TGC GCC GTT CAA G ccc ggg TCA GTA CGC AAA GAT TGC CGT TGC
NCLIV_050270 (ToxoDB)		NcSAG2	SAG2D	gca tgc ACC GAT GTT CAA CAA GCG A aag ctt GTC TGA GTT GCA GGT GCC TA
NCLIV_033230 (ToxoDB)		NcSAG1	Ncp-29	gca tgc ACC TGT GAC AAC GAA GAG AAA T aag ctt CCC ATC CGT GCC GCA AAC
EU200939.1 (Ncbi)	*Sarcocystis* neurona	SnSPR1	SPR1	gca tgc TTC GTG GCC TTC GTG ATC GTG A aag ctt GGC CAC GCA GGT CAC CAG CA
GQ851951.1 (Ncbi)		SnSAG1	SAG1	gca tgc TCC GCC AGA GTG TCC CTT GT aag ctt TAG GCG CGG AGC CGG CGC
GQ851958.1 (Ncbi)		SnSAG4	SAG4	gca tgc ATG TTA CGT GCG ACA GTG TTA C aag ctt AGG AGG CGA GGC GGG AAT ATT
GQ386974.1 (Ncbi)		SnSAG2	SAG2	gca tgc GGC ATA GCA GCA GTT ATT ATT aag ctt CAA CAC TGT GAG AGA TGC GAG T

3.2.2. Multi-sequence alignments

Once a gene was identified, it was compared using the Basic Local Alignment Search Tool (BLAST) (NCBI) to find similar orthologues between *Neospora*, *Toxoplasma* and *Sarcocystis spp*. Orthologues with the lowest identities were chosen, to eliminate cross-reactivity. The coding and predicted protein sequence between the selected target and the orthologue were aligned to compare conserved / polymorphic regions. Multi-sequence alignments on the gene targets and their predicted protein sequences that shared similar orthologues between *Neospora*, *Toxoplasma* and *Sarcocystis* were performed as per section 2.2.2. Multi-sequence alignments. The presence of large conserved regions could lead to cross-reactivity at a later stage. If excessive conservation between the two genes was observed, only the fraction of the gene that showed least homology was chosen. If the whole gene showed too high homology, the gene was discarded, and a new gene was selected.

3.2.3. Primer design for recombinant proteins

Primer design was used to amplify local DNA sequences, where lowest amino acid conservation and highest polymorphisms were seen. Once the least conserved region was chosen, a hydrophobic/hydrophilic plot was created using ProtScale software (http://web.expasy.org/protscale) (Gasteiger E. *et al.,* 2005). The ProtScale software allows the investigation of information in the form of a 2-D plot about the selected protein and predicts hydrophobicity scales based on amino acids (Gasteiger E. *et al.*, 2005). The ProtScale analysis was conducted to increase the likelihood of the protein designed being hydrophilic which would make the recombinant protein more soluble, and would assist during the purification process from bacterial proteins.

The predicted protein sequence was next checked against the corresponding nucleotide sequence within the genome, ensuring that the coding sequence contained only exons (coding regions) and no introns. A sequence translation tool http://www.ebi.ac.uk/Tools/st/ that reads nucleic acid sequences and outputs three forward and three reverse translations was used, showing the reading frames (Li *et al.*, 2015).

Gene-specific primers of a maximum of 22 base pairs were designed spanning the least hydrophobic and least conserved regions. A restriction site analysis was performed using zero cutters NEBcutter V2.0 (http://nc2.neb.com/NEBcutter2) to cross-check the restriction sites available for cloning PCR amplicons in the pQE-30 vector system (Qiagen). NEBcutter enables the sequences to be checked for non-overlapping reading frames using the *E. coli* genetic code, and checks the sites for restriction enzymes that only cut the sequence (Vincze *et al.*, 2003). Appropriate restrictions sites were added to the 5' end of the primers ensuring that the gene of interest could be cloned into the expression plasmid. The difference (delta Tm) in primer melting temperature between the forward and reverse primers was kept within 5°C. If the difference was higher than 5°C, the primer was cut in length / adjusted to meet the criteria. The maximum primer length including restriction site was kept at 28 base pairs and minimum at 22 base pairs.

3.2.4. PCR reactions

Primers designed for recombinant protein production were used to amplify target sequences by PCR as in section 2.2.4. PCR reactions and primer testing PCR were initially tested using a temperature gradient (45 ºC – 65 ºC) to derive the optimal temperature. PCR products were purified and quantified as in section 2.2.5.3. DNA purification and PCR clean-up system and 2.2.8. DNA quantification

3.2.5. Cloning into vector pGEM-T easy

Target products (inserts) were cloned into the pGEM®-T Easy Vector System (Promega, Hollow Road, Madison, USA) as per section 2.2.6. Cloning of target DNA

3.2.6. Mini-prep of plasmid DNA

The plasmids were purified using QIAprep® Spin Miniprep Kit (QIAGEN) as per section 2.2.6.1. Miniprep of plasmid DNA and glycerol stocks were generated as in section 2.2.7. Generation of control DNA using glycerol stocks

3.2.7. Sequencing and sequencing analysis

DNA was sequenced using Eurofins MWG operon sequencing service as per section 2.2.9. Sequencing and sequencing analysis, to confirm that no nucleotide errors had occurred during the PCR process.

3.2.8. Restriction enzyme digest of insert and vector pQE-30

A restriction digest was conducted to remove the gene of interest from the pGEM T-easy construct for further processing. The digest uses restriction enzymes to cut the target insert to prepare the plasmid DNA for subcloning. A restriction digest was also performed on the pQE-30 / 31 QIAexpress pQE vector system to prepare the vector for ligation with the gene of interest to obtain a linear form. The pGEMT-Easy constructs were digested using appropriate restriction enzymes to cut out the correct target (**Table 3. 4.** Recombinant target genes, restriction enzymes and expected coding sequences.**)**. The pQE vector was digested to linearize it, ready for cloning. Approximately, 3µl of buffer 10x NEBbuffer (CutSmart 100) was used, and 1µl of each restriction enzyme (e.g. Hindlll and PstI) was added. Distilled water was added to make the final volume up to 30µl. The digest was incubated at 37°C at 300 rpm for 90 minutes in an Eppendorf ThermoMixer® (Thermofisher). The vector was incubated for 45 minutes at 37°C at 300 rpm in an Eppendorf ThermoMixer®, after which 0.5µl SAP (Shrimp Alkaline Phosphatase) was added and incubated further for 45 minutes. Following the incubation period, 6µl of the digests was run on a 1% Agarose gel using 3µl of a 1kb ladder (NEB) to confirm the presence of the insert/vector. The vector and plasmid were purified as per section 2.2.5.3. DNA purification and PCR clean-up system

3.2.9. Cloning in vector pQE-30

The gene fragments of interest were sub-cloned into the linearized QIAexpress pQE vector system. The pQE vector system was chosen because of its high-level expression of 6x His-tagged protein in *E. coli*. The QIAexpress pQE vectors are based on the T5 promoter transcription-translation system, containing two *lac* operator sequences which increase *lac* repressor binding ensuring efficient binding and efficient repression of the T5 promoter. Protein synthesis was effectively blocked in the presence of high level of *lac* repressors (*lacI*^{q} gene) ensuring more enhanced stability of the cytotoxic constructs. Moreover, the pQE plasmids contain two strong transcriptional terminator 't_0' from phage lambda (Schwarz et al., 1987) and T1 from the rrnB operon of *E. coli*, which prevents read-through transcription and ensures the stability of the expression construct. The vector pQE-30/31 containing the 6xHis affinity tag at the N-terminus of the protein of interest was chosen. The N-terminal tag constructs are 2-4 times more efficient in protein production than proteins with C-terminal tags, and are easier to prepare as only the 5'end of the open reading frame must be ligated as the pQE series provide termination codons in all three frames. Moreover, using 6xHis tag facilitates easy binding of the protein on metal-chelating surfaces, such as Ni-NTA constructs columns for protein purification.

Briefly, ligation was performed by ligating 3µl of the digested plasmid DNA to 1µl of the digested pQE-30 vector using 1µl of 10x buffer (Promega, UK), 1µl of T4 DNA polymerase and 4µl of dH_2O. Following overnight incubation at 4°C, 1µl of the ligation reaction was used to transform 25µl of high-efficiency competent JM109 cells (≥1 × 108cfu/µg DNA) (Promega, UK) using manufacturer's instructions, except using LB-broth medium instead of SOC medium to culture the bacteria. A successful transformation was confirmed using prepared LB agar plates containing 100µg/ml ampicillin. Single colonies were screened by PCR using appropriate primers (**Table 3. 1)**, and samples were sent for sequencing to confirm that the constructs had the correct open reading frame as per section 2.2.9. Sequencing and sequencing analysis Glycerol stocks were generated as per section 2.2.7. Generation of control DNA using glycerol stocks.

3.2.10. Transformation of competent M15 cells

The plasmid DNA in pQE-30/31 vectors were transfected into M15 (pREP4) competent cell for protein expression. The *E. coli* strain M15 carries the *lacI* gene on the pREP4, producing high levels of the *lac* repressor, which permits high-level expression of the desired protein and efficiently blocks transcription when not induced. A high repressor level is needed if the expressed proteins are known to be toxic to cells. Expression of the recombinant proteins encoded by the pQE vector is rapidly induced by IPTG. The transcripts produced by the host cell RNA polymerase transcribes the sequence of interest and translates it into the recombinant protein. The double operator system ensures tight control at the transcriptional level.

Briefly, 1µl of plasmid was transferred to 100µl M15 (pREP4) and kept on ice for 20 minutes. The mixture was heat shocked at 42°C for 1 minute and transferred to ice for 2 minutes. To the transformation mix, 500µl of LB-broth was next added and incubated for 1hr at 37°C. Approximately, 100 µl was plated out on LB-agar plates (containing 25ug/ml kanamycin and 100ug/ml ampicillin) and incubated overnight. A single colony was incubated overnight at 37°C in a shaking incubator (200 rpm) in LB-broth (25ug/ml kanamycin and 100ug/ml ampicillin). Glycerol stocks were generated as per section 2.2.7. Generation of control DNA using glycerol stocks

3.2.11. SDS Page gel electrophoresis

Recombinant proteins were visualised using a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Each recombinant protein was prepared with 4x LDS sample Buffer (LSB) (NuPAGE®, Novex) and 10µl of each sample was heated up to 99°C for 5 minutes at 100 g to denature the protein bonds. A NuPAGE® Bis-Tris-Acetate mini gel (4-12%) was assembled in the XCell SureLock™ Mini-Cell Electrophoresis System (Novex®) as per protocol. Briefly, 10µl of the See Blue® Plus 2 Pre-stained Novax ladder and recombinant protein samples were added. MESSDS running NuPAGE buffer 20x was added to the tank compartment until wells were covered with buffer. The gel was run at 200V for 45 minutes. Coomassie Blue safe stain ™ (Invitrogen) was used to detect the proteins on the SDS PAGE gel, and proteins were visualised using ImageQuant Las 4000.

3.2.12. Expression and Solubility of recombinant protein

Recombinant proteins were expressed in *E. coli*. Initially, a small scale expression culture was set up to verify the expression of the recombinant protein. Briefly, 100µl of the overnight culture was added to a 37°C pre-warmed 5ml LB-broth culture (containing 25 ug/ml kanamycin and 100 ug/ml ampicillin). Following 2hrs incubation at 37°C, IPTG (1 mM) was added to induce expression, and the culture was incubated for 4hrs. Approximately 1ml of culture was taken after 2 and 4 hrs post induction to examine protein expression on an SDS PAGE gel (section 3.2.11. SDS Page gel electrophoresis). The leftover 1.5ml culture was used to test protein solubility as per the QIAexpressionist handbook. Briefly, the pellet was re-suspended in 1ml of Sonication Buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazole, 0.25% Tween20). A sample of 75µl was taken out, and 25µl LSB added. The mixture was sonicated to break open the cells using sound waves in a Vibra-Cell processor VCX 500 (Sonics and Materials, Inc). The samples were sonicated for 10 seconds, placed on ice for 20 seconds and sonicated for a further 10 seconds. The sonication mix was centrifuged for 5 minutes at 13000 rpm. 75µl of the supernatant was collected, and 25µl LSB added. The pellet was re-suspended in 900µl PBS, 75µl of the re-suspended mix was transferred into a new 1.5ml tube, and 25µl LSB was added. Proteins were visualised in an SDS PAGE gel to verify the protein solubility. The native form of many proteins can be insoluble (i.e. most membrane proteins). If recombinant proteins were insoluble, the detergents UREA was used to solubilise the recombinant proteins, which allows for easy purification.

Following the small-scale expression, a large scale expression was set up. The expression was the same as the small-scale expression, with the following exceptions. A 1-litre L-broth culture was used. The culture was incubated at 37°C for 5hrs following the addition of IPTG. Protein expression and solubility were verified using SDS PAGE gel. The pellet was stored at -20°C until further use. Pellets were weighted, and 1g of pellet was re-suspended in 5ml sonication buffer and sonicated. The sonicated mixture was centrifuged for 10 minutes at 10,000 g, and either the soluble fraction (supernatant) or insoluble fraction (pellet) were taken for further processing depending on protein solubility.

3.2.13. Purification of recombinant protein

Proteins were purified to remove any unspecific bacterial protein using Thermo Scientific™ HisPur™ Ni-NTA Spin Columns as per manufacturer's instruction. The spin columns enable an effective purification using metal affinity chromatography (IMAC) purification from poly-histidine-tagged proteins. Ni-NTA resins are commonly used for his-tagged-protein purification because of the four metal-binding sites on the chelate, allowing high-binding capacity and low-metal ion leaching. The purification is divided into three stages: 1) Protein binding stage; when the histidine residue will interact with the nickel ion and bind to the Ni-NTA groups on the column matrix. 2) Washing stage; where endogenous protein (especially insoluble proteins) can result in background problems and interact with the Ni-NTA group, and therefore need to be washed out of the matrix using stringent conditions (either by lowering the pH or adding imidazole at a 10-50mM concentration). 3) The protein elution stage; involving the elution of the 6x histidine tagged-protein by increasing imidazole concentration (100-250mM concentration), during which the protein can no longer bind to the nickel ions and will dissociate from the Ni-NTA resin. Soluble proteins were purified under native conditions, whilst insoluble proteins were purified under denaturing conditions.

Briefly, insoluble proteins (pellet) were re-suspended in 0.5ml of Binding buffer (200mM NaH_2PO_4, 200mM Na_2HPO_4, 500mM NaCl, 10mM Imidazole, 8M Urea) to solubilize the insoluble fraction, and incubated at room temperature for 30 minutes (inverted every 10mins). The mixture was centrifuged for 10 minutes at 13000 rpm, and the supernatant (now containing the protein of interest) was removed and equilibrated using an equal volume of Equilibration buffer (20mM Na NaH_2PO_4, 300mM NaCl, 10mM Imidazole, 8M UREA). The proteins were purified using the HisPur Ni-NTA spin columns (Thermo Scientific) as per manufacturer's protocol, with the exception of using six wash steps with buffers for denaturing conditions. Proteins were washed three times using 6 ml wash buffer (20mM Na NaH_2PO_4, 300mM NaCl, 25mM Imidazole, 8M UREA), and three times with 6 ml wash buffer (20mM Na NaH_2PO_4, 300mM NaCl, 50mM Imidazole, 8M UREA). Proteins were eluted three times using 3 ml elution buffer (20mM Na NaH_2PO_4, 300mM NaCl,

250mM Imidazole, 8M UREA). After each step, the elutions were collected and analysed on an SDS PAGE gel for protein content (section 3.2.11. SDS Page gel electrophoresis).

3.2.14. Protein preparation for polyclonal production

Proteins were prepared for antibody production in rabbits that was carried out by an external commercial company 'ORYGEN Antibodies'. Requested recombinant protein conditions were as follows: a minimum of 4 aliquots of 0.25ml (150 µg) per protein per rabbit, and a UREA concentration of less than 6M UREA. To achieve these conditions, various methods were implemented.

3.2.14.1. Protein dialysis

Recombinant proteins were dialysed to 4 M UREA using Slide-A-LyzerTM G2 Dialysis Cassette (Thermo Scientific) with a 10K molecular weight cut off (MWCO), as per manufacturer's instructions. The cassette processes samples for low molecular weight contaminant removal, buffer exchange, desalting and concentration, and works by the diffusion of molecules from a high to a lower concentration until an equilibrium is reached using a semi-permeable membrane. Larger molecules (i.e. recombinant protein) will not pass through the pores, while smaller molecules will diffuse across the membrane. Performing serial dialysis using buffers with decreasing concentration of solutes prevents the osmotic pressure from swelling the membrane. The membranes are composed of low-binding regenerated cellulose which enables maximum sample recovery as well as maintaining maximum sample purity. Briefly, protein elutions were pooled together and dialysed in 4M UREA in distilled water of at least 300 times the volume of the sample for 2hrs at room temperature. The 4M buffer was changed, and the recombinant proteins further dialysed overnight at room temperature. Protein samples were recovered from the cassettes and stored at -20°C.

3.2.14.2. Concentrating recombinant protein

The recombinant proteins were concentrated using the Vivaspin 6 columns (GE Healthcare Bio-Sciences) at a 10,000 Molecular weight cut off (MWCO) for maximum recovery, according to manufacturer's instructions. Briefly, concentrators were rinsed

with deionised water as the membranes contained traces of glycerine and sodium azide, which could interfere with the antiserum production. Recombinant proteins were added to the concentrator and centrifuged for 25 minutes at 6000rpm. The concentrated protein was removed and stored at -20°C.

3.2.14.3. Determining protein concentration

The protein concentration was verified using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Thermo Scientific), using manufacturer's instructions. Protein concentrations were determined by comparing the protein samples to that of the assay response of a known standard concentration (bovine serum albumin) based on a standard curve. The kit was a detergent-compatible formulation of Bicinchoninic acid (BCA), and was used for the detection and quantitation of proteins. The method combines the reduction of Cu+2 to Cu+1 by a protein with a 'highly sensitive and selective colourimetric detection of cuprous cation (Cu+1) using a unique reagent containing bicinchoninic acid' (Thermo Scientific). The reaction will turn purple by chelation of two BCA molecules with one cuprous ion, exhibiting a strong absorbance at 562nm. The colour formation is due to the number of peptide bonds, and the presence of amino acids (cysteine, cystine, tryptophan and tyrosine) with BCA. Standards were prepared according to the Pierce™ BCA Protein Assay using one BSA ampule of 2mg/ml. Recombinant proteins were diluted to 2M UREA (working reagent of the BCA assay) and prepared with the BCA working reagents (WR) according to protocol.

Briefly, the total volume WR was determined using the standard formula (*standards + *unknowns) x (*replicates) x (volume of WR per sample) = total volume of WR required. Using the microplate procedure, 200 µl of WR was required per reaction. The WR was prepared by mixing 50 parts of reagent A with 1 part of reagent B (50:1). Approximately, 25µl of each standard and recombinant protein samples were added into a 96-Well microplate and mixed with 200µl of WR. Plates were mixed thoroughly for 30 seconds and incubated for 30 minutes at 37°C. The absorbance at an OD of 562nm was measured using a plate reader (Dynex Technologies Headquarters, USA), and a standard curve was plotted using the average blank-corrected for each standard

versus its concentration in µg/ml. The standard curve was then used to determine the protein concentration of each sample.

3.2.15. Western blot analysis

A western blot (or immunoblot) was performed to identify reactivity and cross-reactivity among *Neospora*, *Toxoplasma*, *Sarcocystis neurona* recombinant proteins with positive and negative cattle and sheep immune sera (**Table 3. 1)**. Briefly, recombinant proteins at a concentration of 3ng/ml and positive controls (*Neospora caninum* water-soluble antigen fraction (NAF) of 25 µg/ml and *Toxoplasma* water-soluble antigen fraction (TAF) of 27 µg/ml were separated using a 12% SDS-PAGE gel as per section 3.2.11. SDS Page gel electrophoresis. The proteins were transferred to a nitrocellulose blotting membrane (AmershamTM ProtranTM Premium 0.45µm, GE Healthcare, Life science, Germany), using XCell IITM Blot Module (Invitrogen) for 60 minutes at 30V using the standard protocol. Following overnight incubation at 4°C in 1x Novex® Tris-glycine transfer buffer, membranes were blocked in 10ml of 4% Marvel (non-fat dried milk) for 1 hour at room temperature. Following each 1hr incubation, membranes were washed three times with wash buffer (Tween 80-10ml, 500 mM NaCl, 10xPBS-200ml and dH2O). Positive and negative *Neospora* / *Toxoplasma* / *Sarcocystis tenella* bovine / ovine immune serum were used (**Table 3. 2**).

The positive/ negative immune sera were prepared in 10ml diluent buffer, and the blots were incubated for 1hr at room temperature (**Table 3. 2**). Following a further wash with wash buffer, the membranes were incubated with the secondary conjugate (rabbit anti-bovine IgG HRP or rabbit anti-goat IgG HRP, ThermoFisher Scientific) in 10 ml diluent buffer for 1hr at room temperature (**Table 3. 2**). Following this wash, protein bands were detected using the SuperSignalTM West Pica Chemiluminescent Substrate Kit (ThermoFisher Scientific) enhanced with HRP substrate for low-picogram-level detection. Membranes were incubated with 5mls of SuperSignal West Pico Luminol/Enhancer solution and 5mls of the SuperSignal West Pico Stable Peroxide Solution for 5 minutes, and proteins were visualised using ImageQuant Las 4000.

TABLE 3. 2. WESTERN BLOT CONDITIONS AND DILUTIONS FOR VARIOUS ANIMAL SERA USED IN THIS STUDY.

1º Antibody	Primary sera/ antibody dilution	Secondary conjugate	Conjugate dilution
Neospora +ve bovine serum	1: 200	Rabbit anti-bovine HRP	1: 1000
Toxoplasma +ve bovine serum	1: 200	Rabbit anti-bovine HRP	1: 1000
Sarcocystis tenella +ve ovine serum	1: 200	Rabbit anti-goat HRP	1: 1000
Rabbit Pre-immune sera (RA0355-RA0368)	1: 200	Goat anti-rabbit HRP	1: 1000
Polyclonal Rabbit anti-*Neospora* (NcSAG1)	1: 4000	Goat anti-rabbit HRP	1: 1000
Polyclonal Rabbit anti-*Neospora* (NcSRS2)	1: 4000	Goat anti-rabbit HRP	1: 1000
Polyclonal Rabbit anti-*Toxoplasma* (TgSRS2)	1: 4000	Goat anti-rabbit HRP	1: 1000

Chapter 3.3 Results

3.3.1. Identification of genus-specific proteins

For each target chosen, the protein sequence alignment showed conserved and polymorphic regions between *Neospora*, *Toxoplasma* and *Sarcocystis neurona* (**Figure 3. 1**). Results from the sequence alignment showed that primers were designed from a polymorphic region and conserved regions were avoided as much as possible (**Figure 3. 1**). Results showed an optimum temperature of 65°C for each PCR primer pair. A successful amplification was observed from all *Toxoplasma* designed primers TgSAG1, TgSAG2, TgSAG3 and TgSRS2 (**Figure 3. 2** and **Table 3. 1)**. Successful amplification of the *Neospora* genes was observed for rNcGRA7, rNcSAG1 and rNcSRS2. However, no successful amplification was observed for NcSAG2 (**Table 3. 3**). The only successful amplification for the *Sarcocystis* gene observed was SnSAG2, whereas SnSPR1, SnSAG1 and SnSAG4 showed no successful amplification (**Table 3. 3).**

```
Neospora-NCLIV_033250    TIPDEHYPATSKAFRVGCKAG---KNVCLLNVYVQSRESEVIGQVAHCAY 195
Toxo-TGME49_233480       SVPESQYPANAKSFRVGCRHNTKTGNTCMLTIHVEPRDPAVERQEARCSY 199
                         ::*:.:***.:*:*****: .    *.*:*.::*:.*:. *  * *:*:*

Neospora-NCLIV_033250    SSNVRLRPITVNPENNGVTLICGPDGKAFPDDYMNHHCTELDECKERPYS 245
Toxo-TGME49 233480       TENSTLPKIFVTKDSNTMTLACGPHGAPMPESYTENYCSTPDTCDEKPFT 249
                         :.*  *  * *. :.* :** ***.* .:*:.* :::*:  * *.*:*::

Neospora-NCLIV_033250    AVFPGFSSSFWTGEASGVAGATLTIPKDQFPSTAQTIYLGCTGHPDDK-- 293
Toxo-TGME49_233480       SVIPGYLSKWFFGDPKSPLGAMLRIPPEQIPSSPQIIYLGCTGPTDGEGP 299
                         :*:**: *.:: *:...  ** * ** :*:**:.* ******* .*.:

Neospora-NCLIV_033250    QVTCVVPVNIEEVAKPAGAGSNPGGGSQPDQSSEKRDGEQVNKGKPPTGG 343
Toxo-TGME49_233480       KYNCTVPVQLG--------GEDPSEGSRPG-----------------GG 323
                         : .*.***::         *.:*. **:*.                  **

Neospora-NCLIV_033250    SGGATTGKQNASQNAKDKGETGGENGDSPVLRGDACDELPSYVALSAASL 393
Toxo-TGME49_233480       SGG---GKRGGGQ-----GGGGGLAG-SDSRQGSARHSLPSGMALSASAL 364
                         ***   **:...*     *  **  * *   :*.* ..*** :****::*
```

FIGURE 3. 1. EXAMPLE OF PROTEIN ALIGNMENT BETWEEN *NEOSPORA* AND *TOXOPLASMA* PROTEIN CODING REGION, * (ASTERISK) INDICATES SINGLE CONSERVED AMINO ACID BETWEEN THE TWO PROTEIN SEQUENCES: (COLON) INDICATES CONSERVATION BETWEEN GROUPS OF STRONGLY SIMILAR PROPERTIES, (PERIOD) INDICATES CONSERVATION BETWEEN GROUPS OF WEAKLY SIMILAR PROPERTIES, (RED) START/END OF PRIMER DESIGN.

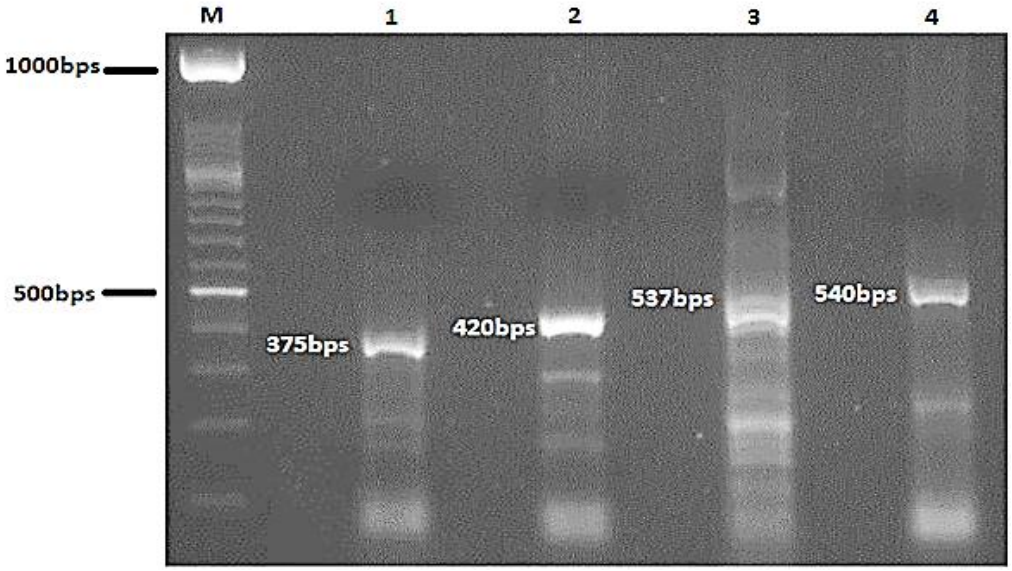


FIGURE 3. 2. PCR AMPLIFICATION OF TARGET GENES IN AN AGAROSE GEL FOR *T. GONDII* PCR AMPLICONS FOR CLONING OF GENES FOR RECOMBINANT PROTEIN CONSTRUCTS. M REPRESENTS A 100 BASE PAIR HYPER LADDER MARKER (BIOLINE), 1- TGSAG1, 2- TGSAG2, 3- TGSAG3, 4- TGSRS2. ESTIMATED BAND SIZE TO THE LEFT OF EACH BAND.

3.3.2. Generation of expression clones

Amplicons from the PCR reaction of the correct amplicon size were successfully purified and cloned into pGEMT-easy (**Figure 3. 3**). Four *Toxoplasma* genes, three *Neospora* genes and one *Sarcocystis* gene were successfully cloned into pGEM T-easy. Sequencing results demonstrated the correct gene was cloned, and no base pair changes occurred during PCR amplification (**Table 3. 3** and **Table 3. 4**). A successful restriction digest was performed on all pGEM T-easy clones using the relevant restriction enzymes as bands of the correct size were observed (**Figure 3. 4** and **Table 3. 3**). Results showed a successful restriction digest of the pQE-30 and pQE31 vector using the restriction enzymes: SpHI and HindIII, SpH1 and PstI, BamHI and Xmal, and KpnI and Xmal (**Figure 3. 5**). Recombinant proteins were successfully sub-cloned into pQE-30 and pQE-31 vectors. Screening and sequencing results revealed no base pair changes during the sub-cloning into the pQE vectors.

TABLE 3. 3. RECOMBINANT PROTEIN PRODUCTION, SHOWING THE REPRESENTATIVE STEPS TAKEN FOR THE DEVELOPMENT OF EACH PROTEIN

Genes	PCR	Cloning & sequencing	Digest	Cloning & sequencing	Transformation in *E. coli*	Protein expression	Protein solubility	Protein purification and Dialysis	Western blots	Protein concentration (µl/ml)	Used for antibody production
Tests		**pGEMP T-Easy**		**pQE-30**	**M15 (pREP4)**	**Expressed**	**Soluble/ Insoluble**	**4 M Urea**			
NcSAG1	✓	✓	✓	✓	✓	✓	In-Sol	✓	No cross-reactivity	5678	✓
NcSRS2	✓	✓	✓	✓	✓	✓	In-Sol	✓	No cross-reactivity	2283	✓
NcGRA7	✓	✓	✓	✓	✓	✓	In-Sol	✓	No cross reactivity	762	×
NcSAG2	×	×	×	×	×	×	×	×	×	×	×
TgSAG1	✓	✓	✓	✓	✓	✓	In-Sol	✓	No cross reactivity	261	×
TgSAG2	✓	✓	✓	✓	✓	✓	In-Sol	✓	Cross reactivity	749	×
TgSAG3	✓	✓	✓	✓	×	×	×	×	×	×	×
TgSRS2	✓	✓	✓	✓	✓	✓	In-Sol	✓	No cross reactivity	3396	✓
SnSPR1	×	×	×	×	×	×	×	×	×	×	×
SnSAG1	×	×	×	×	×	×	×	×	×	×	×
SnSAG2	✓	✓	✓	✓	✓	✓	In-Sol	✓	No reactivity	100	×
SnSAG4	×	×	×	×	×	×	×	×	×	×	×

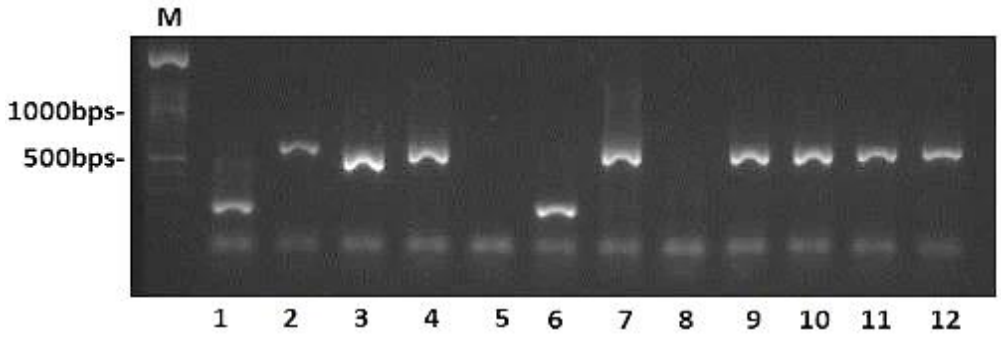


FIGURE 3. 3. EXAMPLE OF A COLONY SCREEN PCR OF 12 PGEMP T-EASY CLONES OF NCSAG2. M REPRESENTS A 100BPS MOLECULAR LADDER, AND 1-12 REPRESENT A DIFFERENT CLONE OF NCSAG2. CLONES 3,4,7,9,10,11,12 CONTAIN THE GENE OF INTEREST.

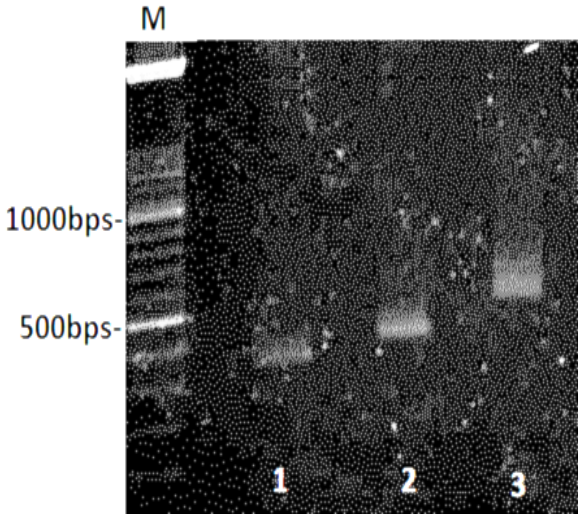


FIGURE 3. 4. EXAMPLE OF A RESTRICTION DIGEST OF *TOXOPLASMA* RECOMBINANT PROTEINS OF WHICH 1-TGSAG1, 2-TGSAG2, 3- TGSRS2 AND M REPRESENTS A 100BPS MOLECULAR WEIGHT LADDER.

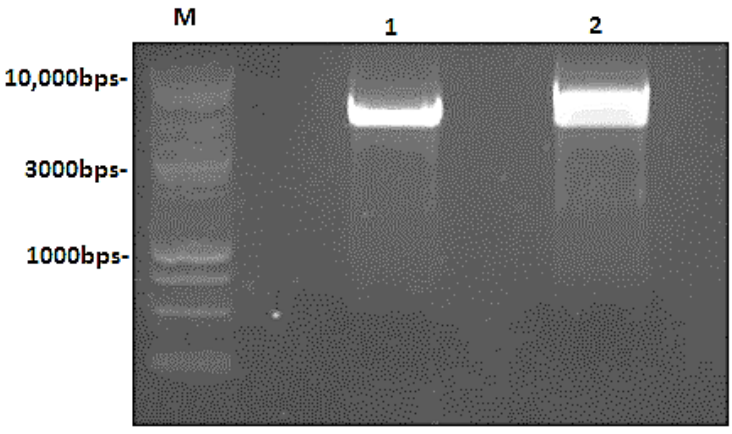


FIGURE 3. 5. EXAMPLE OF A RESTRICTION DIGEST OF PQE-30 VECTOR USING THE RESTRICTION ENZYMES 1= SPHI AND HINDIII AND 2= SPHI AND PSTI. M REPRESENTS A 1KBPS MOLECULAR WEIGHT LADDER.

TABLE 3. 4. RECOMBINANT TARGET GENES, RESTRICTION ENZYMES AND EXPECTED CODING SEQUENCES.

Name	Restriction enzyme	Vector	Expected Coding sequence (bps)	Expected Protein (aa)	Protein Molecular weight (kDa)
TgSAG1	SpHI HindIII	PQE-30	375	124	12.8
TgSAG2	SpHI HindIII	PQE-30	420	140	14.8
TgSAG3	SpHI HindIII	PQE-30	537	164	18.9
TgSRS2	SpHI PstI	PQE-30	540	180	19.2
NcGRA7	BamHI XmaI	pQE-31	576	191	19.9
NcSRS2	KpnI XmaI	pQE-31	1057	354	37.2
NcSAG2	SpHI HindIII	PQE-30	498	166	18.4
NcSAG1	SpHI HindIII	PQE-30	480	160	16.8
SnSPR1	SpHI HindIII	PQE-30	471	157	17.9
SnSAG1	SpHI HindIII	pQE-30	366	122	13.3
SnSAG4	SpHI HindIII	pQE-30	460	158	16.4
SnSAG2	SpHI HindIII	pQE-30	468	156	15.9

3.3.3. Expression and purification of recombinant proteins

Results showed the successful expression of plasmid vectors containing the target gene sequence using *E. coli* strain M15 (pREP4) (**Table 3. 3** and **Figure 3. 6**). All recombinant proteins were shown to be insoluble when expressed and were effectively solubilized using 8M UREA (**Figure 3. 6** and **Table 3. 3**). All recombinant proteins were successfully purified. An example of a successful purification can be seen in **Figure 3. 7**. Proteins were dialysed to a concentration of 4M UREA. Various concentrations of each protein were observed. Concentrations of recombinant proteins ranged from 5678 µg/ml to 100 µg/ml.

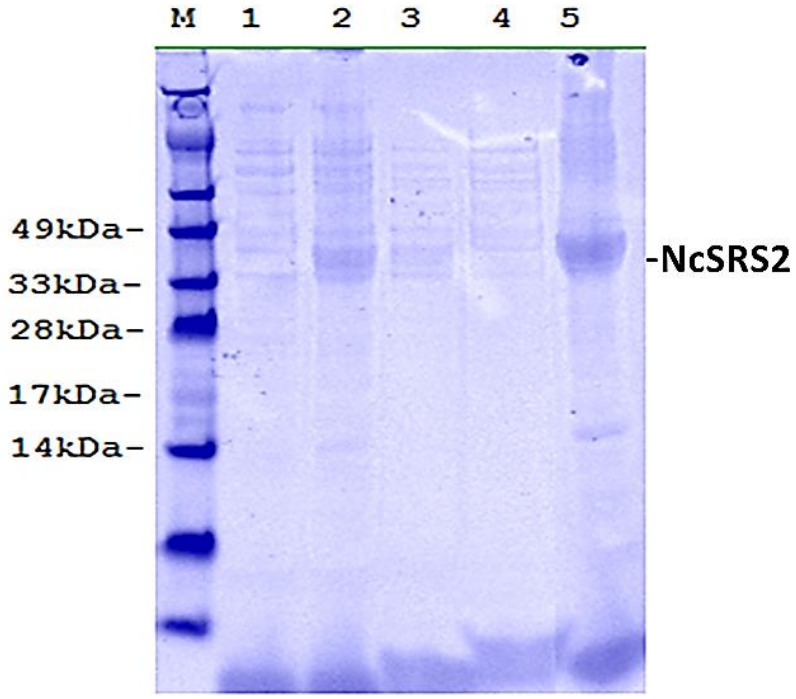


FIGURE 3. 6. SDS PAGE GEL SHOWING EXPRESSION AND SOLUBILITY OF RECOMBINANT PROTEIN NCSRS2. M REPRESENTS THE MOLECULAR WEIGHT LADDER (SEEBLUE PLUS2 PRE-STAINED STANDARD). 1= UN-INDUCED (UN-EXPRESSED), 2= INDUCED (EXPRESSED), 3= PRE-SONICATED FRACTION. 4= SUPERNATANT (SOLUBLE FRACTION) AND 5= PELLET (INSOLUBLE FRACTION) POST SONICATION.

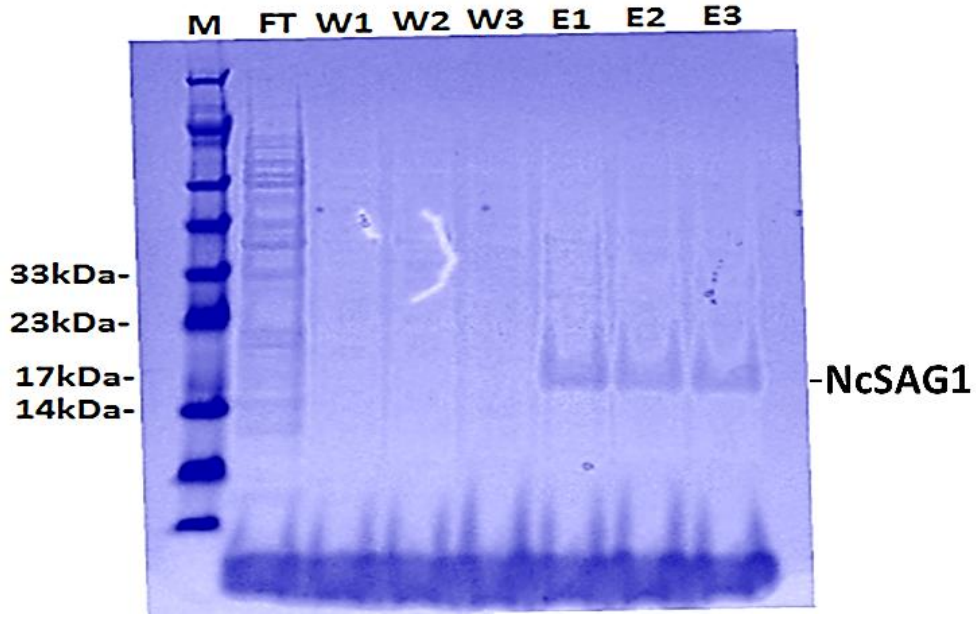


FIGURE 3. 7. PROTEIN PURIFICATION USING 8M UREA OF RECOMBINANT PROTEIN NCSAG1 (16KDA). M REPRESENTS THE MOLECULAR WEIGHT LADDER (SEEBLUE PLUS2 PRE-STAINED STANDARD), FT= REPRESENTS THE FLOW THROUGH, W1, W2, W3 REPRESENT THE WASHING STEPS 1, 2 AND 3 OF THE COLUMN AND E1, E2, E3 REPRESENT THE ELUTION STEP 1, 2, AND 3 OF THE RECOMBINANT NCSAG1 FROM THE COLUMN.

3.3.4. Recombinant proteins cross-reactivity testing using Western blot analysis

The western blot analyses showed that when using '*Neospora* positive bovine' immune serum, reactivity with *Neospora* antigen fraction and *Neospora* recombinant proteins (rNcSAG1, rNcSRS2 and rNcGRA7) were observed (**Figure 3. 8 1A**). No reactivity with '*Neospora* negative bovine' immune serum was observed with *Neospora* antigen fraction and recombinant proteins rNcSAG1, rNcSRS2, rNcGRA7, rTgSRS2, rTgSAG1, rTgSAG2 and rSnSAG2 (**Figure 3. 8 1B**). Moreover, *Toxoplasma* and *Sarcocystis* recombinant proteins (rTgSRS2, rTgSAG1, and rSnSAG2) and *Toxoplasma* antigen fraction showed no reactivity with the '*Neospora* positive bovine' serum (**Figure 3. 8 1A**). Reactivity with rTgSAG2 was observed with *Neospora* positive bovine serum (**Figure 3. 8 1A**).

Similar results were observed for the western blot analyses with '*Toxoplasma* positive bovine' serum, which showed reactivity with the *Toxoplasma* antigen fraction and the recombinant protein rTgSRS2, rTgSAG1 and rTgSAG2 (**Figure 3. 8 2A**). No reactivity with either *Neospora* antigen fraction and/or rNcSAG1, rNcSRS2, rNcGRA7 or rSnSAG2 was observed (**Figure 3. 8 2B**). Using *Toxoplasma* negative bovine serum, no reactivity with the *Toxoplasma*, *Neospora* and *S. neurona* recombinant proteins was observed (**Figure 3. 8 2B**). Results using *S. tenella* positive ovine serum showed no reactivity with the rSnSAG2, rNcSAG1, rNcSRS2, rNcGRA7 and rTgSAG1. On the other hand, reactivity with *Toxoplasma* rTgSAG2 and rTgSRS2 was observed.

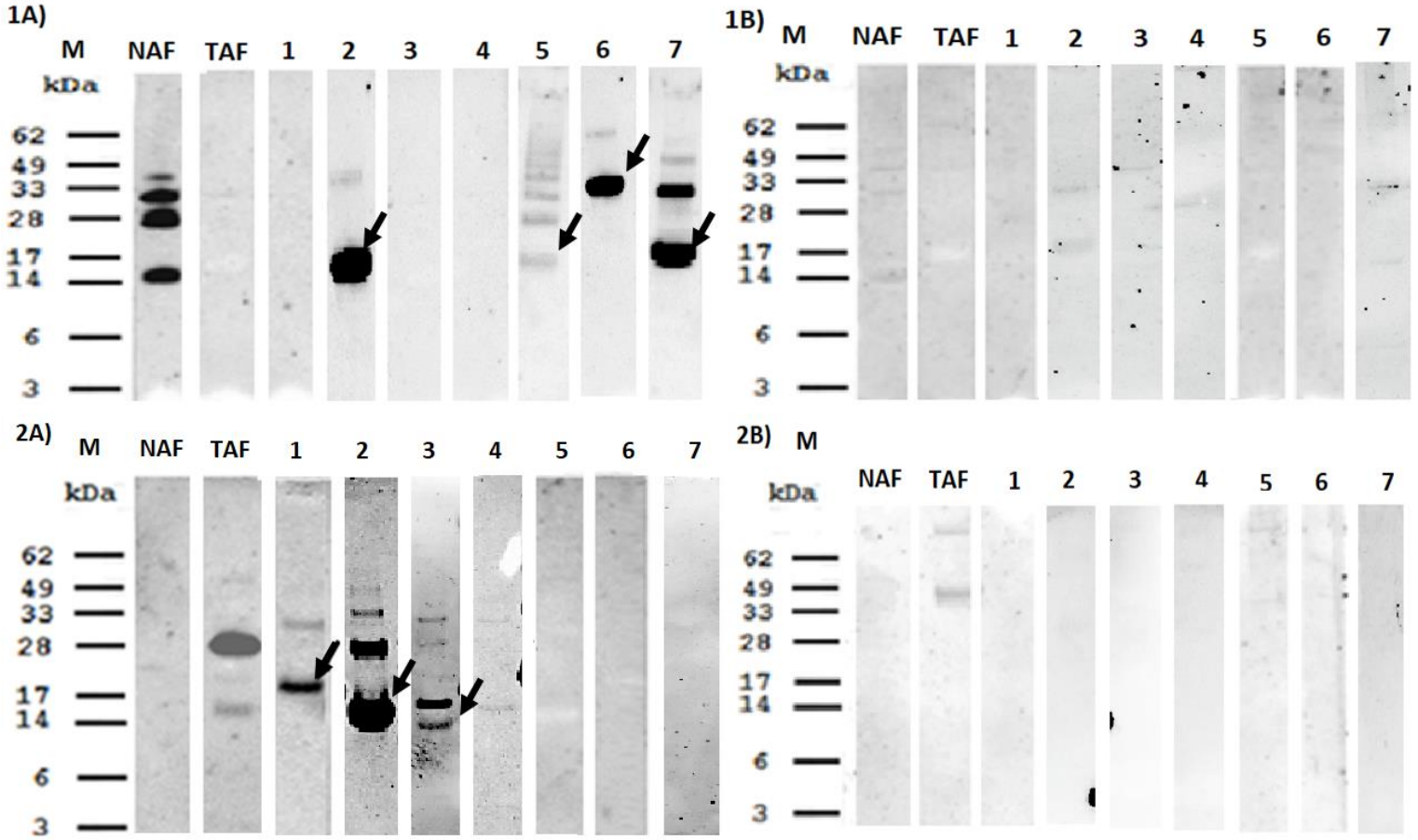


FIGURE 3. 8. REACTIVITY AND CROSS-REACTIVITY TESTING BY WESTERN BLOT FOR RECOMBINANT PROTEIN NAF= *NEOSPORA* ANTIGEN FRACTION AND TAF= *TOXOPLASMA* ANTIGEN FRACTION. 1= RTGSRS2, 2= RTGSAG2, 3= RTGSAG1, 4= RSNSAG2, 5= RNCSAG1, 6= RNCSRS2 AND 7= RNCGRA7. 1A) WESTERN BLOT TESTED USING *NEOSPORA* POSITIVE BOVINE SERUM 1B) *NEOSPORA* NEGATIVE SERUM 2A) *TOXOPLASMA* POSITIVE BOVINE SERUM 2B) *TOXOPLASMA* NEGATIVE BOVINE SERUM.

Chapter 3.4. Discussion

Neosporosis, toxoplasmosis and sarcocystosis are common diseases worldwide, and losses to livestock farms caused by these protozoans are substantial. Accurate monitoring programs are required to distinguish and confirm the presence or absence of protozoal parasites, in order to estimate the levels of infection within a herd and to adopt the most relevant disease control strategy. Specific diagnostic tests are therefore needed to implement these accurate control strategies. This Chapter discussed the production of genus-specific recombinant proteins against *T. gondii, N. caninum* and *Sarcocystis* spp. that will be used in the production of genus-specific antibodies for the diagnosis of protozoan infections using IHC.

In this study, various surface membrane proteins including SAG genes (TgSAG1, TgSAG2, TgSAG3, NcSAG1, NcSAG2, SnSAG1, SnSAG2, SnSAG4, SnSPR1), SRS genes (TgSRS2, NcSRS2), and one dense granular gene (NcGRA7) were chosen as targets for recombinant protein production for each coccidian species, *N. caninum*, *T. gondii* and *Sarcocystis neurona*. Surface membrane proteins play a crucial role in host cell recognition, adhesion and invasion into host cells, and are thus suitable targets for use as diagnostic reagents (Burg *et al.*, 1988; Chahan *et al.*, 2003; Kim & Boothroyd, 2005; Reid *et al.*, 2012). While the surface of each protozoan parasite is dominated by surface antigens, a greater repertoire of SRS genes was observed in *Neospora* (227 genes) compared to *Toxoplasma* (104 genes) (Reid *et al.*, 2012; Wasmuth *et al.*, 2009). Having protein epitopes localised on the outer membranes of the parasite makes them an ideal candidate for IHC diagnosis, as it can enable maximum recognition of each parasite. Even though *Toxoplasma*, *Neospora* and *Sarcocystis* spp. share common gene similarities, genetic differences on surface antigens and surface related sequences and how they mediate the initial host-cell interaction have been identified and can be used to generate genus-specific recombinant proteins as they have shown to be more specific to each individual parasites limiting cross-reactivity (Sohn *et al.*, 2011).

These small numbers of unique host-specific gene differences are potential diagnostic targets, and could be used to avoid cross-reactivity between the differing protozoan

parasites (Kim & Boothroyd, 2005; Sinnott *et al.*, 2017; Uzeda *et al.*, 2013). The SAG, SRS and GRA genes were additionally chosen as target proteins based on their expression during different life cycle stages. Various genes are known to be expressed either during acute (tachyzoite) stage (i.e. TgSAG1, TgSAG2, TgSRS2, NcSAG1 and NcSAG2) (Dong *et al.*, 2012; Huang *et al.*, 2002; Kim & Boothroyd, 2005; Lekutis *et al.*, 2000; Reid *et al.*, 2012; Wang & Yin, 2014), or chronic (bradyzoite) stages (NcGRA7, NcSRS2 and TgSAG3) (Aguado-Martínez *et al.*, 2010; Khanaliha *et al.*, 2014; Sinnott *et al.*, 2017) or during both stages (Reid *et al.*, 2012). Choosing genes that express protein in either or both tachyzoites, bradyzoites and merozoites would be ideal, as it enables recognition of each protozoan parasite during various stages. Only the most polymorphic regions of the proteins, based on the Tg and Nc amino acid sequence comparisons were expressed, to avoid recognition of antibodies recognising conserved epitopes.

Initially, this study wanted to produce *S. tenella* recombinant proteins as it is a common pathogenic species in sheep in the UK (Schock *et al.*, 2012). However no information was available on the genome of *S. tenella* at the time of primer design. Only information on *Sarcocystis neurona* surface gene families (SnSAG) and (surface protein) SPR1 were available, and therefore these have been selected as diagnostic targets due to their highly immunogenic nature, displayed on the merozoites surface and their potential homology in related *Sarcocystis* parasites (Howe *et al.*, 2005; Zhang & Howe, 2008). Primers were designed to amplify the surface gene targets for *S. neurona* and were initially tested using *S. tenella* DNA, yet this was shown to be unsuccessful, and indeed was only successful using *S. neurona* DNA. Using a *S. neurona* gene as a target, a potential of cross-reactivity with the SAG genes with other closely related *Sarcocystis* species could be possible, as surface membrane proteins orthologues seem to be uniquely expressed by the *Sarcocystis* genus (Howe *et al.*, 2005; Sohn *et al.*, 2011; Tenter *et al.*, 1991; Zhang & Howe, 2008).

During this study, it was shown that four target genes from *Toxoplasma*, three target genes from *Neospora* and only one *Sarcocystis neurona* target gene were successfully amplified and cloned into the various vectors (pGEMP T-easy and pQE-30). However, not all gene targets were successfully expressed in the bacterial expression system M15

(pREP4). The expression of membrane proteins, such as SAG and SRS may be difficult due to their toxicity in *E. coli*. It is well known that using a bacterial expression system to express membrane proteins or proteins that interact with DNA can cause problems due to the inability to maintain the expression construct during cell growth (Samuelson, 2011; Wagner *et al.*, 2007). Consequences can include cellular stress responses that can lead to the formation of inclusion bodies, toxicity and limited growth (Akiyama, 2009; Carrio & Villaverde, 2002; Samuelson, 2011; Wagner *et al.*, 2007). Even though the M15 strain is specifically used in the expression of toxic proteins due to its higher levels of repressors (Samuelson, 2011), problems due to expression and subsequent membrane toxicity might still arise. Many genes can severely interfere with the survival of *E. coli* cells, and can lead to the death of bacteria causing significant growth limitation and drastically decrease expression of recombinant proteins (Saida *et al.*, 2006). Due to the expression of toxic proteins, low concentration levels were observed, and some recombinant proteins (rNcGRA7, rTgSAG1, rTgSAG2, rSnSAG2) showed lower concentrations compared to others (rNcSAG1, rNcSRS2, rTgSRS2).

Sonication of expressed proteins allows the disruption of cells, to help the release and recovery of high protein yields from *E. coli* cells in either the soluble or insoluble fraction of the lysate (Benov & Al-Ibraheem, 2002; Feliu *et al.*, 1998). Insoluble proteins can be contained in inclusion bodies, which depend on the nature of the protein, the host cell and the level of expression (Bornhorst & Falke, 2000; Samuelson, 2011). In our study, it was shown that each recombinant protein was localised in the insoluble fraction of the lysate, which may have been due to the distribution of hydrophobic residues on the surface of the protein and level of expression. High levels of expression of many recombinant proteins can led to the formation of these highly aggregated proteins (inclusion bodies) (Palmer & Wingfield, 2012). It was shown by the Structural Genomic Centre that 50% of all prokaryotic proteins were insoluble when expressed in *E. coli* (Edwards *et al.*, 2000). Moreover, it was observed that previously expressed *N. caninum,* and *T. gondii* proteins in *E. coli* were contained in inclusion bodies, which resulted in a loss of antigenicity due to misfolding (Debache *et al.*, 2009; Nigro *et al.*, 2001). Due to the insolubility of the recombinant proteins, refolding of proteins into their active states with a denaturant was necessary to obtain

a biofunctional and soluble protein (Bornhorst & Falke, 2000; Dong *et al.*, 2012; Palmer & Wingfield, 2012). The urea denaturant solubilises and refolds insoluble proteins that are contained in inclusion bodies, and dissolves the protein precipitation, formed by overexpression in *E. coli* (Yang *et al.*, 2011). Even though most membrane proteins produce insoluble forms, they can be re-folded to recover their antigenicity and regain their activity. However, the refolding process is highly inefficient (5 to 8%) and time-consuming (Chen *et al.*, 2001). It was demonstrated by Chen *et al.* (2001) that immunoreactivity was recovered after refolding of the recombinant protein TgSAG1. Using immobilised metal-affinity chromatography for the purification of proteins with 6 x polyhistidine affinity tags allowed a successful purification of the seven recombinant proteins, as it enabled purification of insoluble proteins (Bornhorst & Falke, 2000). The purification was performed using multiple wash steps to extract highly pure proteins, avoiding nonspecific bacterial protein that could lead to non-specific reactivity during western blot analyses. Our results showed a low recovery concentration of most recombinant proteins developed, and thus proteins had to be concentrated to obtain a suitable concentration for downstream use.

Cross-reactivity and reactivity were tested using western blot analysis to check which protein could be taken forward for polyclonal antibody production. The recombinant proteins rNcSAG1, rNcSRS2 and rNcGRA7, and the *Neospora* antigen fraction, were recognised by the serum from cattle positive with *Neospora*. No reactivity of either recombinant proteins or *Neospora* antigen fractions was noticed with a serum that was negative for *Neospora*. Various genes of *N. caninum* are known to be homologues to those of closely related species. In this study, rNcGRA7, rNcSRS2 and rNcSAG1 were shown to have only a 33%, 43% and 51% identity in amino acid content to those of equivalent *T. gondii* proteins, respectively, explaining their specificity in the western blot analyses (Dong *et al.*, 2012). Moreover, during the western blot analysis, no recognition of the *Neospora* recombinant proteins or *Neospora* antigen fraction was observed when using serum from cattle positive with *Toxoplasma*. These results indicate that the recombinant protein fractions do not show any cross-reactivity with *Toxoplasma* serum, and could, therefore, be used for the development of polyclonal antibodies. Similar results were obtained during the study by Chahan *et al.*, (2003), who showed that there was no cross-reactivity with the truncated rNcSAG1 fraction

with sera from mice experimentally infected with *T. gondii*. Comparable results were also shown in other studies, showing no cross-reactivity between recombinant surface antigen NcSRS2 and NcSAG1 with anti-*T. gondii* cat, cattle and mouse sera (Bjorkman & Hemphill, 1998; Dong *et al.*, 2012; Howe *et al.*, 1998; Nishikawa *et al.*, 2002; Schares *et al.*, 2000). Moreover, it was shown that antibodies generated against NcGRA7 showed no cross-reactivity against *T. gondii* in immunofluorescence analysis (Aguado-Martínez *et al.*, 2010). Even though all three rNcSAG1, rNcSRS2 and rNcGRA7 proteins have shown no cross-reactivity with *Toxoplasma* positive serum, only rNcSAG1 and rNcSRS2 were taken forward for polyclonal antibody production, due to their higher expression and concentration levels compared to rNcGRA7.

Similar results were obtained in the western blots for the *Toxoplasma* recombinant proteins. The recombinant proteins rTgSAG1, rTgSAG2 and rTgSRS2, and the *Toxoplasma* antigen fraction were all recognised by *Toxoplasma* positive cattle serum. No reactivity was observed with *Toxoplasma* negative cattle serum. Moreover, no reactivity of *Toxoplasma* recombinant proteins rTgSAG1 and rTgSRS2 or *Toxoplasma* antigen fractions was observed with cattle serum positive for *Neospora*, suggesting that the *Toxoplasma* recombinant protein are specific to *Toxoplasma*. The study by Kimbita *et al.*, (2001) showed no cross-reactivity with the homologue TgSAG1 fraction with sera from mice experimentally infected with *N. caninum*. Similar results were also seen in the study by Howe *et al.*, (1998), who demonstrated that even though TgSAG1 and TgSRS2 are homologues of their *N. caninum* counterparts and share structural similarities, the levels of sequence identity of TgSRS2 and TgSAG1 were not sufficient to elicit a cross-reactive antibody response with *Neospora*. Even though the *Toxoplasma* recombinant proteins (rTgSAG1 and rTgSRS2) showed no cross-reactivity, only rTgSRS2 was chosen for polyclonal production due to high expression and high concentration levels compared to rTgSAG1.

However, a faint reactivity with the recombinant protein rTgSAG2 was observed with *Neospora* positive serum, and therefore this recombinant protein was not taken forward for further use. This may have been due to the way the rTgSAG2 had been expressed and refolded following re-solubilisation, which could have resulted in

antigenicity and/or structural changes, hence explaining the cross-reactivity towards *Neospora* positive sera (Khanaliha *et al.*, 2012; Macedo *et al.*, 2013; Sabaj *et al.*, 2010; Wu *et al.*, 2009). It could have also been due to TgSAG2 containing conserved epitopes that are also present in *Neospora*. The recombinant protein rTgSAG2 has been reported to be specific to *T. gondii,* and no cross-reactivity in experimental and natural infections were observed with *N. caninum* using various serological diagnoses (Bjorkman & Uggla, 1999; Gondim *et al.*, 2017; Huang *et al.*, 2004; Huang *et al.*, 2002; Santana *et al.*, 2012). In the present study rTgSAG2 fraction showed cross-reactivity with *Neospora* positive cattle serum and was discarded for polyclonal antibody production. This could have been due to the different methods or regions of expression, solubility or the way the recombinant protein was refolded. Various authors have used various expression systems (Khanaliha *et al.*, 2012; Macedo *et al.*, 2013; Sabaj *et al.*, 2010; Wu *et al.*, 2009) and this may result in changes in the property (antigenicity) and structure of the protein. Structural changes can be observed depending on whether the protein was produced as a soluble or insoluble protein and the way it has been refolded, which influences the immune activity of the protein (Wu *et al.*, 2009), and that only part of the protein regained immune activity (Wu *et al.*, 2009).

Cross-reactive antigens between *Neospora* and *Toxoplasma* have been previously observed by IHC using tissues of naturally and experimentally infected animals when using antibodies produced from whole parasite antigen (Dubey *et al.*, 1996; Gondim *et al.*, 2017; Silva *et al.*, 2007). A polyclonal rabbit anti-*Neospora* serum cross-reacted with *T. gondii* infected tissue sections from mice (Barta & Dubey, 1992). Other studies have shown that when using whole *N. caninum* soluble antigen, cross-reactivity with *T. gondii* positive mouse and cat sera was observed (Nishikawa *et al.*, 2002). When using crude *Neospora* soluble antigen, a high level of serological cross-relativity with *T. gondii* can be observed, and it was shown that *N. caninum* antibodies recognized *T. gondii* antigens (Silva *et al.*, 2007). However, in our study for the development of *Neospora* and *Toxoplasma* recombinant proteins, only fractions of each antigen targets and only the most polymorphic region of each gene targets were selected to limit cross-reactivity.

The *S. neurona* (rSnSAG2) recombinant protein used in the present study showed no cross-reactivity with *S. tenella* positive ovine serum, which suggests that the similarity of the surface membranes between *S. neurona* and *S. tenella* are less homologous than initially expected. Since *Sarcocystis neurona* was thought to exhibit homology with other closely related *Sarcocystis* species at a genetic level, it was also expected to display similarity with *Sarcocystis tenella* (Howe *et al.*, 2005). The lack of cross-reactivity could simply indicate that the *S. neurona* target used was uniquely expressed in *S. neurona*, explaining this minimal homology between the surface antigens within *Sarcocystis* spp. Therefore, the *S. neurona* recombinant protein was not taken forward for further work.

The use of such molecular techniques for the development of recombinant proteins has greatly expanded diagnostic analysis as an alternative to using whole parasite antigens, isolated from cultivated parasites (Gondim *et al.*, 2017). This chapter reports the development of specific recombinant proteins from target genes of which two *Neospora* and one Toxoplasma recombinant protein were taken forward for the production of rabbit polyclonal anti-serum.

Chapter 4.

Chapter 4.1. Improved IHC diagnosis: genus-specific antibody development

4.1.1. Introduction

Pathogens, such as the protozoan parasites *Neospora*, *Toxoplasma* and *Sarcocystis* spp., can cause a wide spectrum of similar clinical symptoms in the same animal species, and cannot be easily distinguished solely on clinical findings (Washington, 1996). Each of these protozoan parasites are known to have antigenic and structural similarities as previously discussed in chapters 1, 2, and 3 and hence diagnosis may prove difficult (Bjerkas *et al.*, 1994; Bjerkas *et al.*, 1984; Dubey *et al.*, 1988a). Currently, there are no effective treatments (vaccines or drugs) available for those diseases, and in order to implement effective control measures, rapid, reliable, sensitive and most of all specific diagnostic tools are needed (Sahinduran, 2012). Yet the identification of these parasites is particularly difficult, and the diagnosis of abortions is difficult to achieve, even in well-established diagnostic laboratories (Oporto *et al.*, 2006).

Cattle are the principal intermediate hosts for neosporosis, with abortions being the main clinical signs of the disease (Dubey & Schares, 2006; Dubey & Schares, 2011; Dubey *et al.*, 2007; Shaapan, 2016). However, other ruminant, such as sheep can also be infected by *N. caninum,* causing the same clinical disease. The same can be seen in toxoplasmosis being mainly associated with abortion in sheep, yet *T. gondii* can be found in other ruminants, including cattle (Buxton *et al.*, 2007; Dubey & Lindsay, 2006; Dubey & Schares, 2011). Moreover, various *Sarcocystis* spp. have also been reported in both sheep and cattle and various clinical signs are seen depending on pathogenicity of the *Sarcocystis* spp., involved (Dubey & Lindsay, 2006).

The diagnosis of abortions in ruminants is routinely based on gross pathology, histopathology and specific labelling of the parasites by IHC using various tissue

samples from the aborted fetuses. Formalin fixation and paraffin embedding enables the long-term preservation of pathological samples in histopathology for the diagnosis of disease (Lin *et al.*, 2009). Formalin fixation preserves morphology and cellular details if fresh tissue is no longer available due to decomposition of the tissue (Kokkat *et al.*, 2013; Lin *et al.*, 2009). Histopathology analysis using H&E is an important diagnostic tool for detecting protozoal lesions, cysts and inflammation caused by the parasites in foetal brain and muscle tissues. However, diagnosis of clinically infected animals is difficult even in well-preserved tissues due to lesions not being always associated with the presence of the parasites, and to the structural similarities between these protozoan parasites (Boger & Hattel, 2003; Dubey & Lindsay, 2006). An apicomplexan-like protozoa found in the brain of an aborted bovine foetus does not necessarily indicate *N. caninum* as the cause of abortion, as other protozoan parasites, such as *Toxoplasma* or *Sarcocystis* could be present as they have also been reported to infect cattle (Amdouni *et al.*, 2017; Dubey, 1986; Filho *et al.*, 2017; Hassig *et al.*, 2003).

IHC is a more sensitive and specific method for the diagnosis of the presence of protozoan than conventional histology. IHC uses specific antibodies for the detection of parasite-antigens compared to solely staining the nuclei of the cell and cytoplasm during H&E (Cabral *et al.*, 2009; Pescador *et al.*, 2007a; Ramos-Vara *et al.*, 2008; Uzeda *et al.*, 2013). IHC allows identification of one or several immunogenic epitopes allowing visualisation of the parasites within tissue sections, and hence the distribution of the pathogen in the tissues (Haines & West, 2005; Ramos-Vara *et al.*, 2008). Polyclonal antibodies (pAb) are known to recognise several epitopes of the same pathogen, giving it a higher sensitivity, whereas monoclonal antibodies (mAb) bind to a specific epitope making them more specific (Ramos-Vara *et al.*, 2008). Despite having higher sensitivity than mAb in IHC examinations, *N. caninum* pAb raised against whole parasite lysates can cross-react with other cyst forming parasites, such as *T. gondii* and *Sarcocystis* spp. (McAllister *et al.*, 1996b; Peters *et al.*, 2000; Van Maanen *et al.*, 2004). The mAb raised against whole parasite lysates have also been widely used in diagnostic laboratories to detect *Neospora* infections. However the reliability, cost and usefulness of mAb in naturally-infected tissues are debatable, and

needs to be further evaluated for its use in IHC analyses (Aguado-Martínez *et al.*, 2010; Sohn *et al.*, 2011; Uzeda *et al.*, 2013).

Antibodies raised against protozoan antigens that cross-react with other morphologically similar cyst-forming parasites, such as between *Neospora* and *Toxoplasma*, have previously been identified (Gondim *et al.*, 2017; Liao *et al.*, 2005; McAllister *et al.*, 1996b). *Neospora* and *Toxoplasma* share a high degree of antigenic homology, and thus share similar conformational structures, responsible for the cross-reactivity if unspecific antibodies are used (Howe & Sibley, 1999). Antibodies developed using whole parasite lysates often show cross-reactivity with other protozoan, and hence are often unsuitable for distinguishing between these closely related parasites using IHC (Gondim *et al.*, 2017). Thus using native or total antigens for antibody production can greatly reduce the specificity of a diagnostic test, which can be problematic in adapting adequate control measures, as the correct species cannot be identified. Antibodies that show a high specificity as well as a high sensitivity are needed. Various studies have shown that using specific protozoan proteins in their native and recombinant forms have reduced cross-reactivity with other coccidian parasites (Borsuk *et al.*, 2011; Dong *et al.*, 2012; Gondim *et al.*, 2017; Kotresha & Noordin, 2010; Nishikawa *et al.*, 2001; Sa *et al.*, 2014; Silva *et al.*, 2013; Silva *et al.*, 2012; Uzeda *et al.*, 2013). The development of recombinant proteins can be used to produce antibodies that are specific, and are able to distinguish between closely related species.

Once specific antibodies have been developed, they have to be implemented in the diagnostic assay (i.e. IHC) and tested for their functionality and specificity (Bordeaux *et al.*, 2010; Jacobson, 2018; Mattocks *et al.*, 2010; Ramos-Vara *et al.*, 2008). However, there are only few general guidelines for the standardization and validations of the diagnostic assays, and the definition criteria required for assay validation are elusive (Jacobson, 2018). Moreover, the process of implementing these diagnostic assays for diagnostic use on natural samples involves a complex series of steps of assessment and validation of the assay itself (Jacobson, 2018; Mattocks *et al.*, 2010). The standardization procedure of each antiserum will determine optimal conditions (incubation temperature, time, optimum dilution, buffers, retrieval methods and

detection system) depending on the animal tissue, species and fixation method to ensure that each selected antiserum will react with the expected antigen (Ramos-Vara *et al.*, 2008). Once optimum conditions have been determined, the antiserum / antibody will be validated, which is the process of optimizing the test method and determining the performance characteristics of each antiserum / antibody (Bordeaux *et al.*, 2010; Jacobson, 2018; Ramos-Vara *et al.*, 2008).

For most IHC methods, validation of each antiserum also involves the detection of the cross-reactivity potential with unrelated antigen, as well as cross-reactivity amongst various tissues and animal species. Additional validation of the antiserum / antibody can be performed by testing the sensitivity and specificity of the 'gold standard' method of detection via comparison of antibodies for the same target species (Ramos-Vara *et al.*, 2008). Antiserum / antibodies should also be validated for its original intended purpose, i.e. using natural cases for the diagnosis of the target antigen. This chapter shows the use of protozoal recombinant proteins for the development of genus-specific antibodies that can be used as diagnostic tools in ruminant abortion cases. The antiserum and antibodies produced will be standardized and validated using a series of immunohistochemical analyses to test for functionality and specificity of the antiserum for the purpose of diagnosing ruminant infected with protozoan parasites.

4.1.2. Aims and objectives

In this chapter polyclonal rabbit serum against recombinant proteins will be raised for the genus-specific detection of *Neospora* and *Toxoplasma*. The functionality and specificity of the rabbit polyclonal serum will be tested, and the best candidate antigen will be selected and used for monoclonal antibody production. Genus-specific IHC using antiserum and protocols developed will be validated with natural cases from the Moredun pathology and surveillance archive. This will be achieved by:

- Test rabbit pre-immune sera for cross-reactivity and choose the best candidate rabbit for immunisation with recombinant proteins
- Raise rabbit polyclonal sera against recombinant proteins
- Test rabbit polyclonal sera for functionality and specificity
- Compare rabbit polyclonal sera against commercially available antibodies / antiserum
- Testing the genus-specific IHC using polyclonal rabbit antiserum using natural ruminant abortion cases
- Chose the best antigen candidate for monoclonal antibody production

The second aim of this chapter is to raise monoclonal antibodies against *Neospora* and *Toxoplasma* recombinant proteins. Mouse polyclonal serum and hybridoma clones will be tested for functionality and specificity. The best antigen candidates from the polyclonal sera will be selected for the development of the monoclonal antibodies.

- Raise monoclonal antibodies using *Neospora* and *Toxoplasma* recombinant proteins
- Test mouse polyclonal sera for functionality and specificity
- Test hybridoma supernatants for functionality and specificity
- Select best candidate hybridoma clone for antibody production
- Test each monoclonal antibody for functionality and specificity

Chapter 4.2. Materials and Methods

4.2.1. Immunohistochemistry (IHC)

Paraffin-embedded tissue blocks were cut into 4µm sections and placed onto a water bath at 46^0 C. Sections were either placed onto SuperFrost Plus® (Thermo scientific) glass slides for IHC analyses or SuperFrost® slides (Thermo Scientific) coated with albumin solution (5mg Albumin, 50mls dH2O, 50mls glycerol) for H&E. Sections were dried overnight at 37^0 C and then kept in the fridge until use. Prior to use, sections were further incubated at 60^0 C for 30 minutes. Tissues were dewaxed as shown in Error! Reference source not found.. Endogenous peroxidase was quenched by i mmersion of the slides in 3% hydrogen peroxide in methanol for 20 minutes under mixing conditions. Following a wash for 5 minutes in running tap water, slides were assembled with cover plates in sequenza racks and washed three times using Tris-Buffer Saline (TBS) (50ml Tris HCl, 30ml NaCl pH 7.6.) until the wash buffer was fully drained. Various antigen retrieval (AR) methods were used, to 'de-mask' the modification of antigen targets caused by neutral buffer formalin fixation. Following antigen retrieval methods, slides were blocked in 25 % Normal Goat serum or 2.5 % Normal Horse serum for 30 minutes at room temperature (**Table 4. 1**). The primary antibody (**Table 4. 1**) was added to the slides and incubated overnight at 4°C. The following morning slides were washed three times with TBS. The secondary conjugates were applied according to manufacturer's instruction and incubated for 30 minutes at room temperature (**Table 4. 1**).

TABLE 4. 1. REACTION CONDITIONS FOR THE IHC METHOD FOR EACH ANTIBODY AND SECONDARY CONJUGATE USED IN THIS STUDY. MRI= MOREDUN RESEARCH INSTITUTE.

1º Antibody	Primary antibody dilution	Negative control	Secondary conjugate	Binding reagent	Reference
Polyclonal rabbit serum					
Rabbit pre-immune sera	1:2000	Rabbit pre-immune sera	Dako Envision Goat anti-Rabbit HRP	25 % Normal goat serum	This study
Rabbit anti-*Neospora*-NcSAG1	1:2000	Rabbit pre-immune serum Ra361	Dako Envision Goat anti-Rabbit HRP	25 % Normal goat serum	This study
Rabbit anti-*Toxoplasma*-TgSRS2	1:2000	Rabbit pre-immune serum Ra365	Dako Envision Goat anti-Rabbit HRP	25 % Normal goat serum	This study
Rabbit anti-*Neospora*-NcSRS2	1:2000	Rabbit pre-immune serum Ra368	Dako Envision Goat anti-Rabbit HRP	25 % Normal goat serum	This study
Commercial					
Rabbit anti-*T. gondii* IgG antibody (Thermofisher)	1:600	Normal rabbit serum	Dako Envision Goat anti-Rabbit HRP	25 % Normal goat serum	Thermo Fisher
Goat anti- N. caninum polyclonal serum (VMRD)	1:2000	Normal goat serum	ImmPress Horse anti-goat HRP	2.5 % Normal Horse serum	VMRD
Others					
Rabbit anti-*Streptococcus agalactia* III	1:2000	Normal rabbit serum	Dako Envision Goat anti-Rabbit HRP	25 % Normal goat serum	MRI
Moredun in-house					
Polyclonal Rabbit anti-*Neospora* (NC1)	1:1000	Biotinylated goat anti-rabbit IgG	Dako Envision Goat anti-Rabbit HRP	25 % Normal goat serum	MRI (Buxton *et al.*, 1997)
Polyclonal Rabbit anti-*Toxoplasma* (M1/M2-669)	1:1000	Normal rabbit serum	Dako Envision Goat anti-Rabbit HRP	25 % Normal goat serum	MRI (Dagleish *et al.*, 2010)
Polyclonal Rabbit anti-*Sarcocystis* (9953311)	1:80–1:280	Normal rabbit serum	Dako Envision Goat anti-Rabbit HRP	NA	MRI (Dagleish *et al.*, 2010)
Monoclonal antibodies					
Neospora / *Toxoplasma* polyclonal mice serum	1:200	Normal mouse serum	Dako Envision Goat anti-mouse HRP	25 % Normal goat serum	This study
Hybridoma supernatant	1:1	Hybridoma control supernatant	Dako Envision Goat anti-mouse HRP	25 % Normal goat serum	This study
Neospora Monoclonal antibody ME7	1: 500	Mouse IgG	Dako Envision Goat anti-mouse HRP	25 % Normal goat serum	This study

Slides were washed three times with TBS and treated with peroxidase substrate solution (AEC) (Vector®) following manufacturer's instructions. The ACE reaction was stopped by the addition of distilled water after 30 minutes. Slides were counterstained with Mayer's haematoxylin for 2 minutes followed by immersion in Scot's tap water substitute (STWS) for 2 minutes and covered using aqueous ImmunoHistoMount^{TM} until dry. Sections were mounted with a coverslip using permanent Shandon ConsulMount^{TM}. The IHC analysis was performed with at least one positive control tissue (Error! Reference source not found.). Positive control t issues derived from various host species (sheep, cat, dog, mouse, and cattle) contained cysts and/or tachyzoites and/or bradyzoites from experimentally infected animals with *T. gondii*, *N. caninum* and *Sarcocystis* spp. (Error! Reference source not found.**).**

4.2.1. Polyclonal project: Production and screening of rabbit polyclonal sera

4.2.1.1. Screening of rabbit pre-immune sera

Pre-immune sera from fourteen rabbits (RA0355-RA0368) were tested using western blots and IHC. Western blots were performed as in section 3.2.14. (**Chapter 3-Table 3. 2**). Rabbits were selected for the study when no reactivity from their serum with the recombinant proteins or with *Neospora* and/or *Toxoplasma* antigen fractions was observed. Additionally, the rabbit pre-immune serum samples were tested using IHC as per section 4.2.1.Immunohistochemistry (IHC), with no pre-treatment method and at a dilution of 1: 2000 using positive tissue block (Error! Reference source not f ound.**)**. The rabbits were selected based on the least amount of background staining and least amount of parasite labelling with the pre0immune sera.

4.2.1.2. Production of polyclonal sera

Rabbit polyclonal sera were produced by ORGYEN Antibodies (www.orygen.co.uk**)** according to the company's protocol. Briefly, three New Zealand white rabbits were immunised four times every four weeks (week 1, 4, 8, 12) with recombinant proteins at a concentration of 150ug per dose (**Table 4. 2**). The rabbit Ra361, Ra368 and Ra365 were inoculated against recombinant proteins rNcSAG1, rNcSRS2 and rTgSRS2,

respectively for the development of polyclonal rabbit antisera. For each injection, 0.25ml of recombinant protein was emulsified with complete Freund's Adjuvant and administered to each rabbit. Test bleeds were taken after the boosts in week five and nine. Rabbits were exsanguinated one week after the final injection at week 13 (**Table 4. 2**).

TABLE 4. 2. IMMUNISATION SCHEDULE FOR POLYCLONAL ANTISERUM PRODUCTION USING RECOMBINANT PROTEIN RNCSAG1, RNCSRS2 AND RTGSRS2.

Procedure	Week	Rabbit
Pre-immune bleed	1	10ml serum approx.
Immunise (Injection No 1)	1	
Boost (Injection No 2)	4	
Bleed	5	Test bleed
Boost (Injection No 3)	8	
Bleed	9	Production bleed
Boost (Injection No 4)	12	
Bleed	13	Exsanguination

4.2.1.3. Standardization of polyclonal rabbit serum

Each polyclonal sera (anti- *Neospora* NcSAG1, anti- *Neospora* NcSRS2 and anti-*Toxoplasma* TgSRS2) were tested using western blot analysis. Reactivity and specificity were tested against total *Neospora* and *Toxoplasma* parasites lysate, water-soluble fractions and recombinant proteins (rNcSAG1, rNcSRS2 and rTgSRS2) as per section 3.2.15. Western blot analysis.

Polyclonal sera (anti- *Neospora* NcSAG1, anti- *Neospora* NcSRS2 and anti-*Toxoplasma* TgSRS2) were also tested using IHC analysis. First reactivity of each polyclonal antisera were also evaluated using IHC analysis as follow. First optimum dilutions were tested by using the polyclonal rabbit anti-sera with different dilutions (1:100, 1:200, 1:400, 1:500, 1:1000 and 1:2000) to obtain the optimum dilution for each serum/antibody. Next the optimum antigen retrieval method was tested. Antibodies were tested using, no retrieval treatment, heat-induced epitope AR (HIER) and proteolytic / enzyme -induced epitope AR (PIER) until an optimum retrieval method was chosen. Briefly, for the HIER AR method slides were autoclaved at 121°C for 10 minutes using citrate buffer (10mM citric acid, at pH 6.0) and allowed to cool

to 50°C. For the PIER AR method, slides were incubated at 37°C for 10 minutes using 0.1% protease (from Streptomyces griseus, Sigma-Aldrich) in TBS and allowed to cool to room temperature in water. For testing of dilution and antigen retrieval methods, polyclonal anti-*Neospora*-NcSAG1 and anti-*Neospora*-NcSRS2 were tested using *Neospora* positive control dog cases (MP12/929 and 11820) and polyclonal anti-*Toxoplasma*-TgSRS2 was tested using *Toxoplasma* positive control cat cases (Cat brain 1 and MP14/630) (**Appendix I Table VI**). The rabbit pre-immune sera were used as a negative control with each test performed.

Second, specificity was tested as follow. The rabbit polyclonal anti-*Neospora*-NcSAG1, anti-*Neospora*-NcSRS2 and anti-*Toxoplasma*-TgSRS2 were tested using two *Neospora* control cases (MP12/929 and 11820), two *Toxoplasma* control cases (Cat brain 1 and MP14/630) and two *Sarcocystis* control cases (MP15/0847 and MP06/0228) using the optimum dilution and retrieval method (**Table 4. 1. and Appendix I Table VI**). IHC analysis were performed as per section 4.2.1. Immunohistochemistry (IHC).

Thirdly, the polyclonal anti-*Neospora*-NcSAG1, anti-*Neospora*-NcSRS2 and anti-*Toxoplasma*-TgSRS2 were compared to the rabbit anti-*T. gondii* IgG antibody (Thermofisher), goat anti- *N. caninum* polyclonal serum (VMRD) and rabbit anti-*Streptococcus agalactia* III using two *Neospora* control cases (11838 and 11820), two *Toxoplasma* control cases (Cat brain 2 and MP14/630) and two *Sarcocystis* control cases (MP15/0848 and MP06/0228) (**Table 4. 1. and Appendix I Table VI**).

4.2.1.4. Validation of polyclonal antisera using IHC analysis

For the validation of each antiserum, 111 abortion cases from ruminants (85 bovine and 26 ovine samples) were selected, based on availability, complete data sets and previous immunohistochemistry analysis of findings of lesions or cysts consistent with protozoal infections in foetal brain, placenta or muscle tissues from the Moredun pathology and surveillance archive. Tests were performed in order to test specificity of the new diagnostic in comparison to the alternative IHC method using the Moredun in-house antisera and to see if the reagents improve on the IHC assay (**Table 4.1.)**.

One block from each case was selected, and four slides were cut per block. Slides were tested with anti- *Neospora* NcSAG1 serum, anti- *Neospora* NcSRS2 serum, anti-*Toxoplasma* (TgSRS2) serum and anti-*T. gondii* IgG antibody (Thermofisher). Each IHC performed used positive *Neospora* control cases (MP12/929, 11838 and 11820) and *Toxoplasma* control cases (Cat brain 1 and 2) (**Appendix I Table VI**). As a negative antibody control the rabbit pre-immune sera were used (**Table 4. 1.**). Slides were considered positive when labelling of protozoan parasites was observed.

4.2.1.4.1. Statistical analysis

The proportion of positive samples (prevalence) with confidence intervals (95% CI) was calculated for the presence of positive *Toxoplasma* and *Neospora* labelling via IHC analysis for each antiserum / antibody.

A Fisher's exact test was additionally used to test if the two *Neospora* antisera and *Toxoplasma* antisera differed in sensitivity in the number of positive results. The data was analysed using the online software at https://www.graphpad.com/quickcalcs/contingency1.cfm and calculated using the two-tailed value. Differences were considered statistically significant at $P < 0.05$.

4.2.3. Monoclonal project: Production and screening of monoclonal antibodies

Monoclonal antibodies were produced by Antibody Production Services (APS) (http://www.antibodyproduction.co.uk) using recombinant proteins rNcSRS2 and rTgSRS2. Briefly, each recombinant protein at a concentration of 3.5mg/ml was injected into four Balb/c mice using the same immunisation schedule as for the rabbit polyclonal antisera production (**Table 4. 2**).

Mice were exsanguinated, and serum was screened as per company protocol by ELISA to test for reactivity and specificity against *Neospora* and *Toxoplasma* antigens at a concentration of 1µg/ml. *Neospora* and *Toxoplasma* antigens were coated with Carbonate/Bicarbonate Buffer (Na_2CO_3 + $NaHCO_3$) onto Maxisorp plates. Reactions were carried out using wash buffer PBS / Tween 0.2%. An anti-mouse IgG- HRP conjugate was used at a dilution of 1: 2000, and reactions were analysed using a TMB Liquid Substrate System. The cutoff OD values were less than 0.2. Polyclonal mouse sera that were specific by ELISA were additionally screened and tested for specificity using IHC as per section 4.2.1 Immunohistochemistry (IHC) (**Table 4. 1**).

Following mouse serum screening, the spleens of the best two mice that showed highest specificity and sensitivity were prepared, and B- lymphocyte cells were fused with myeloma cells (tumour cell) to develop hybridomas. Parental hybridoma supernatant and sub-clones were tested by ELISA against *Neospora* and *Toxoplasma* antigens at a concentration of 0.5µg/ml, at a cut off OD value of 0.25. As previously, IHC using *Neospora / Toxoplasma / Sarcocystis* positive control slides was used to determine specificity.

For each IHC test, two positive controls and one negative control were used. For the positive controls, the rabbit polyclonal anti- *Neospora* (NcSRS2) / anti- *Toxoplasma* (TgSRS2) sera and the mouse polyclonal anti- *Neospora* (NcSRS2) / anti- *Toxoplasma* (TgSRS2) were used and for the negative control, normal mouse serum was used. Only parental hybridomas that showed reactivity and specificity by ELISA and IHC were selected for limited dilution sub-cloning. For *Neospora* monoclonal antibody production, only 12 parental hybridoma clones positive by ELISA were tested by IHC, and for *Toxoplasma* monoclonal antibody production, all 18 parental clones positive

by ELISA were tested by IHC, due to limited control material available. Each supernatant of the parental clone was tested using *Neospora* and *Toxoplasma* control tissues to identify reactivity and cross-reactivity.

Once a stable cell line was established, hybridoma clones were expanded and purified using protein G affinity to produce the monoclonal antibodies, as per company protocol. Purified antibodies were concentrated to a final concentration of 1mg/ml, and the monoclonal antibody was optimized using IHC. The antibodies were tested using various dilutions; 1:50 (20µg/ml), 1:200 (5µg/ml) and 1:500 (2µg/ml) to derive the best working dilution. Once an optimal dilution was chosen, the antibody was tested for specificity using two *Toxoplasma*, two *Sarcocystis* and two *Neospora* control tissues (Error! Reference source not found.).

Chapter 4.3 Results

4.3.1. Selection of rabbits for immunisation

Western blot results revealed that 4 rabbit pre-immune sera (n= 14) showed reactivity towards *Neospora* and *Toxoplasma* recombinant proteins. No reactivity of the pre-immune sera was observed towards the *Neospora* and *Toxoplasma* antigen (**Table 4. 3**). IHC showed strong background and unspecific labelling in 11 out of 14 sera for the protozoan parasites *Neospora, Toxoplasma* and *Sarcocystis* (**Table 4. 3**). Pre-immune rabbit serum 361 showed no background or unspecific labelling for *Toxoplasma*, *Neospora*, and *Sarcocystis* (**Figure 4. 1**). Pre-immune serum from rabbit 365 showed some degree of labelling for *Toxoplasma*, however, no labelling was observed for *Neospora* and *Sarcocystis* (**Figure 4. 1**). Serum form rabbit 368 showed no labelling for *Toxoplasma* and *Sarcocystis*, but mild labelling was observed for *Neospora* (**Figure 4. 1**). The pre-immune screen test revealed that only three rabbits (Ra361, Ra365 and Ra368) were suitable for polyclonal serum production. Two *Neospora* recombinant proteins rNcSAG1 and rNcSRS2 and one *Toxoplasma* recombinant protein rTgSRS2 were used to immunise rabbit Ra361, Ra368 and Ra365, respectively

TABLE 4. 3. REACTIVITY OF 14 PRE-IMMUNE RABBIT SERA TESTED BY WESTERN BLOTS (WB) AGAINST THE RECOMBINANT PROTEIN, AND *NEOSPORA* AND *TOXOPLASMA* ANTIGEN FRACTION AND TESTED BY IMMUNOHISTOCHEMISTRY AGAINST TISSUE CONTAINING *NEOSPORA*, *TOXOPLASMA* AND *SARCOCYSTIS* PARASITES.

	Rabbit number	WB (+/-ve)	IHC (+/-ve)	Antibody development
1	RA0355	+	+	No
2	RA0356	+	+	No
3	RA0357	-	+	No
4	RA0358	-	+	No
5	RA0359	-	+	No
6	RA0360	-	+	No
7	RA0361	-	-	**Yes**
8	RA0362	+	+	No
9	RA0363	-	+	No
10	RA0364	-	+	No
11	RA0365	-	+ only Toxo	**Yes**
12	RA0366	+	+	No
13	RA0367	-	+	No
14	RA0368	-	+ only Neo	**Yes**

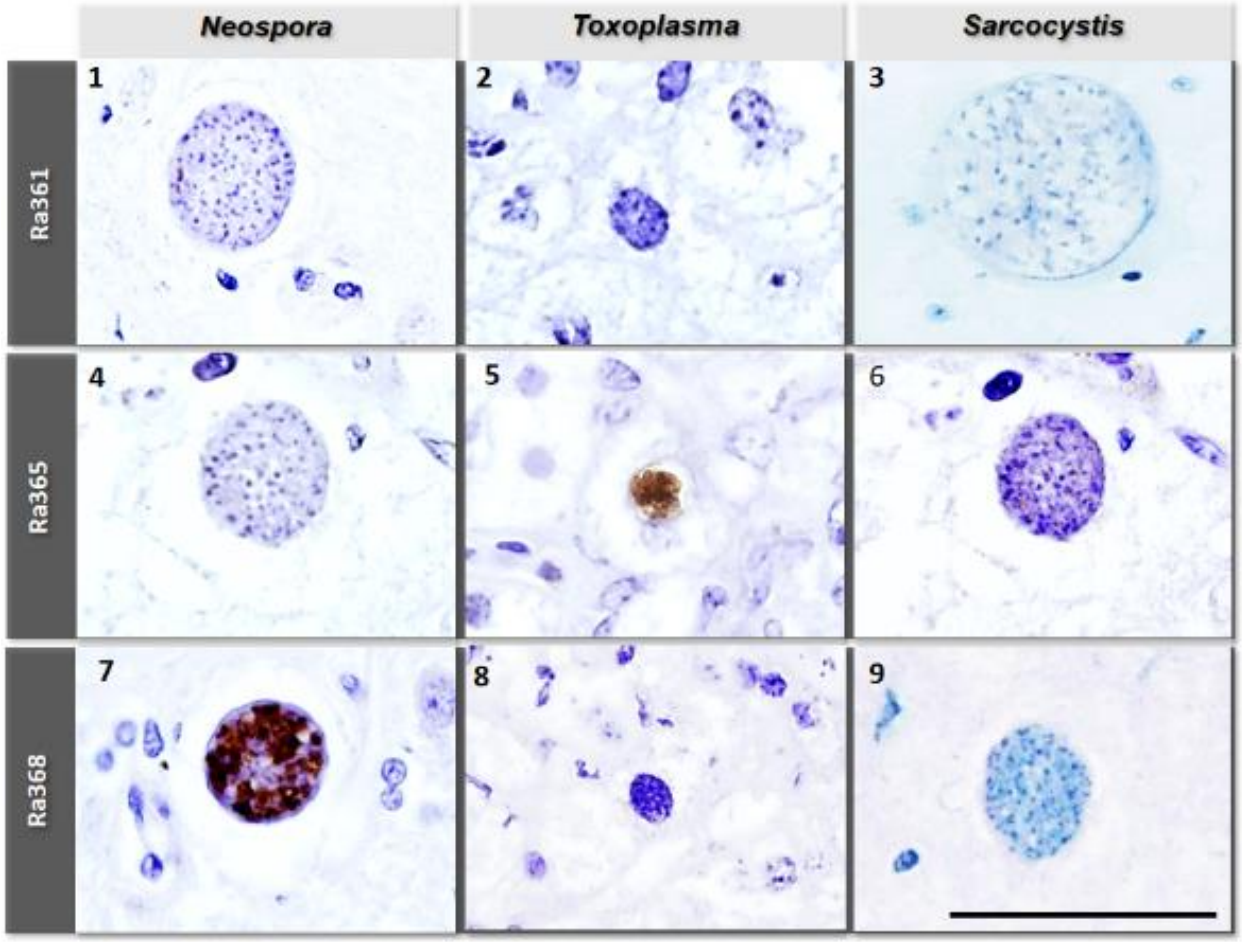


FIGURE 4. 1. IHC ANALYSIS OF PRE-IMMUNE RABBIT SERUM. 1-3: RABBIT SERUM RA361 AGAINST 1- *NEOSPORA* CONTROL TISSUE, 2- *TOXOPLASMA* CONTROL TISSUE AND 3- *SARCOCYSTIS* CONTROL TISSUE. 4-6: RABBIT SERUM RA365 AGAINST 4- *NEOSPORA* CONTROL TISSUE, 5- *TOXOPLASMA* CONTROL TISSUE AND 6- *SARCOCYSTIS* CONTROL TISSUE. 7-9: RABBIT SERUM RA368 AGAINST 7- *NEOSPORA* CONTROL TISSUE, 8- *TOXOPLASMA* CONTROL TISSUE AND 9- *SARCOCYSTIS* CONTROL TISSUE. BAR: 50 µM.

4.3.2. Standardization and validation of rabbit polyclonal sera

4.3.2.1. Analysis of cross-reactivity of polyclonal sera using western blots analysis

Anti- *Neospora* NcSAG1 showed strong reactivity with the rNcSAG1, mild reactivity with rNcSRS2 and NAF and no reactivity with the TAF or rTgSRS2 (**Figure 4. 2**). Anti- *Neospora* NcSRS2 showed strong reactivity with rNcSRS2 and NAF, mild reactivity with rNcSAG1, and no reactivity with TAF or rTgSRS2 (**Figure 4. 2**). Anti- *Toxoplasma* TgSRS2 showed strong reactivity with rTgSRS2, and no reactivity with TAF, NAF, rNcSRS2 or rNcSAG1 was observed (**Figure 4. 2**). As no *Sarcocystis tenella* recombinant protein was produced, no polyclonal antiserum was developed.

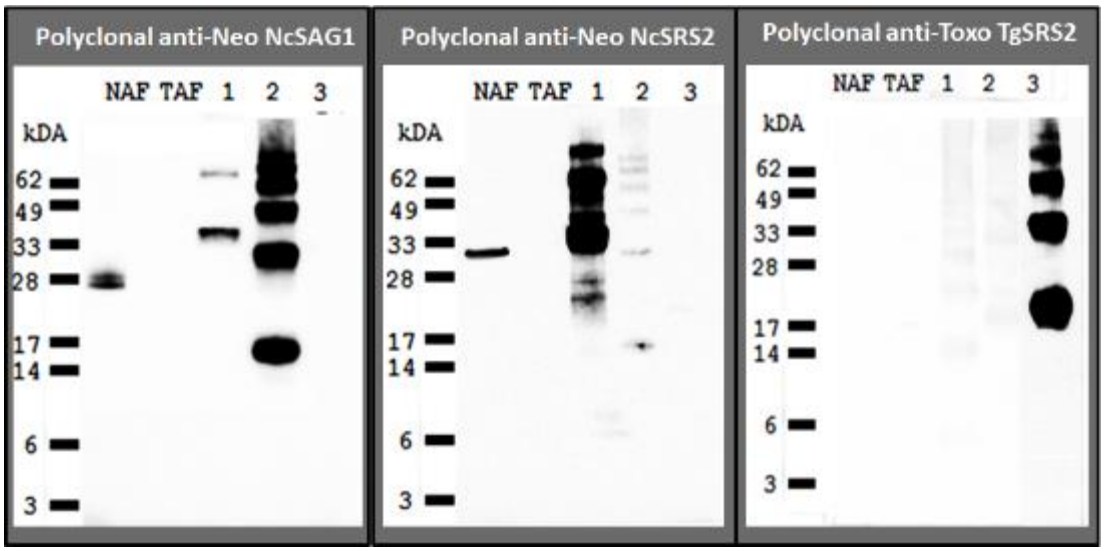


FIGURE 4. 2. WESTERN BLOTS ANALYSIS TO TEST REACTIVITY AND SPECIFICITY OF THE POLYCLONAL RABBIT SERUM NCSAG1, NCSRS2, TGSRS2 AGAINST THE RECOMBINANT PROTEINS, 1- NCSRS2, 2- NCSAG1, 3- TGSRS2 AND ANTIGEN FRACTIONS NAF = *NEOSPORA* ANTIGEN FRACTION AND TAF= *TOXOPLASMA* ANTIGEN FRACTIONS.

4.3.2.2. Verification of functionality of Rabbit polyclonal sera using IHC

IHC results showed labelling of *Neospora* with rabbit anti-*Neospora* (NcSAG1 and NcSRS2) with all dilutions used (1:100, 1:200, 1:400, 1:500, 1: 1000, 1:2000). Similar results were observed with rabbit anti-*Toxoplasma* (TgSRS2), and labelling of *Toxoplasma* parasites with each dilution tested was observed. Results revealed that the negative control (rabbit pre-immune sera) showed unspecific labelling of *Neospora* and *Toxoplasma* when using dilutions of 1:100, 1:200, 1:500. However, no unspecific labelling with pre-immune serum was observed at a dilution of 1: 1000 and 1: 2000 using PIER retrieval.

Additionally, each polyclonal serum was tested using no pre-treatment, a HIER retrieval method (citrate buffer) and a PIER retrieval method (protease) was used, to establish the most efficient method. Results showed that when no pre-treatment was used, the labelling of the protozoan parasites were faint (**Figure 4. 3, Figure 4. 4** and **Figure 4. 5).** Slides treated with HIER showed much stronger labelling, but the microscopic structure of the tissues was altered making the interpretation of results difficult. Slides treated with PIER showed clear and strong labelling of the protozoan parasites, and a well-preserved microscopic structure (**Figure 4. 3, Figure 4. 4** and **Figure 4. 5)**

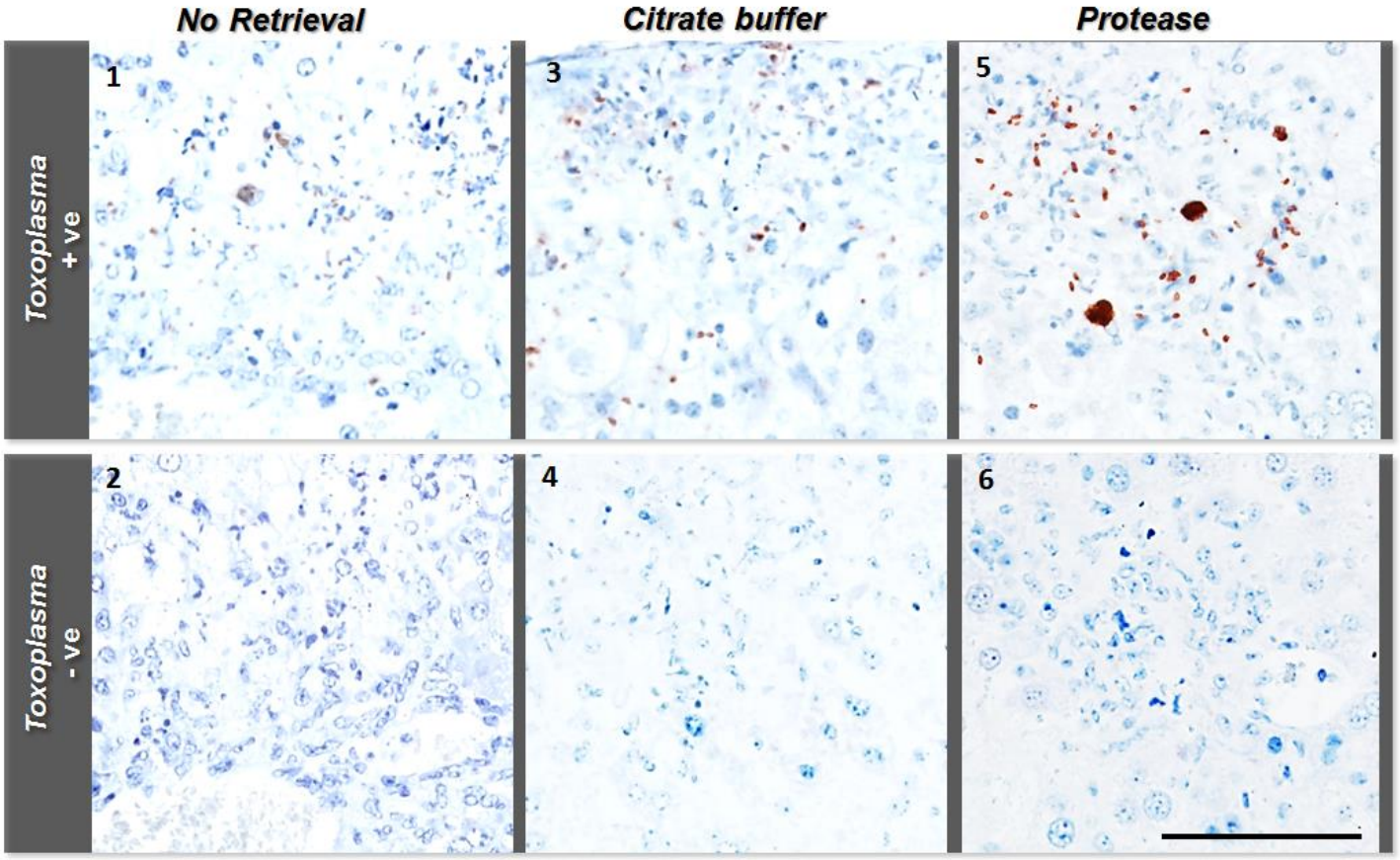


FIGURE 4. 3. IMMUNOHISTOCHEMISTRY VALIDATION OF POLYCLONAL SERUM AT A DILUTION OF 1:2000. 1-2: NO TREATMENT. 1- POSITIVE *TOXOPLASMA* CONTROL TISSUE TESTED WITH RABBIT ANTI-*TOXOPLASMA* (TGSRS2) SERUM, 2- POSITIVE *TOXOPLASMA* CONTROL TISSUE TESTED WITH RABBIT PRE-IMMUNE SERUM RA365. 3-4: HIER TREATMENT. 3- POSITIVE *TOXOPLASMA* CONTROL TISSUE TESTED WITH RABBIT ANTI-*TOXOPLASMA* (TGSRS2) SERUM, 4- POSITIVE *TOXOPLASMA* CONTROL TISSUE TESTED WITH RABBIT PRE-IMMUNE SERUM RA365. 5-6: PIER TREATMENT. 5- POSITIVE *TOXOPLASMA* CONTROL TISSUE TESTED WITH RABBIT ANTI-*TOXOPLASMA* (TGSRS2) SERUM, 6- POSITIVE *TOXOPLASMA* CONTROL TISSUE TESTED WITH RABBIT PRE-IMMUNE SERUM RA365. BAR: 100µM.

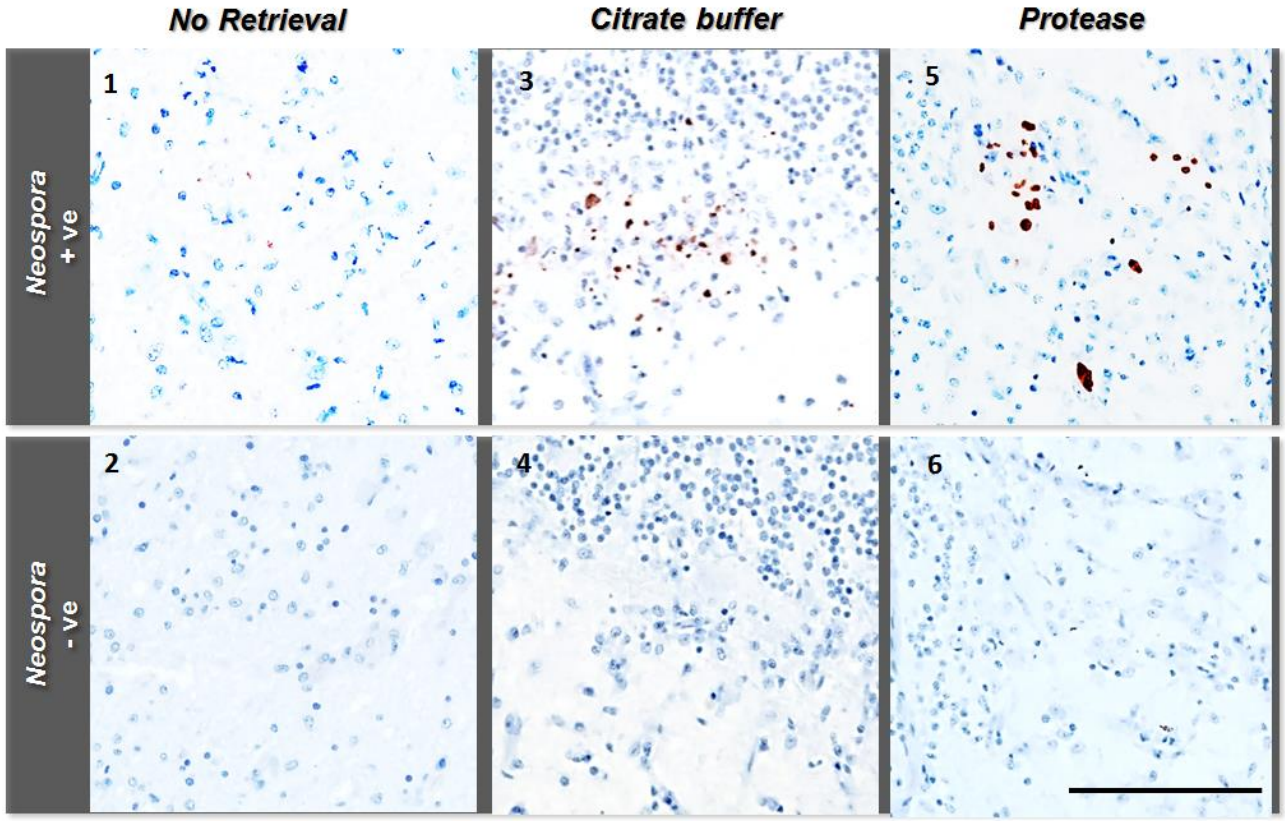


FIGURE 4. 4. IMMUNOHISTOCHEMISTRY VALIDATION OF POLYCLONAL SERUM AT A DILUTION OF 1:2000. 1-2: NO TREATMENT. 1- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT ANTI-*NEOSPORA* (NCSAG1) SERUM, 2- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT PRE-IMMUNE SERUM RA361. 3-4: HIER TREATMENT. 3- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT ANTI-*NEOSPORA* (NCSAG1) SERUM, 4- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT PRE-IMMUNE SERUM RA361. 5-6: PIER TREATMENT. 5- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT ANTI-*NEOSPORA* (NCSAG1) SERUM, 6- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT PRE-IMMUNE SERUM RA361. BAR: 100µM.

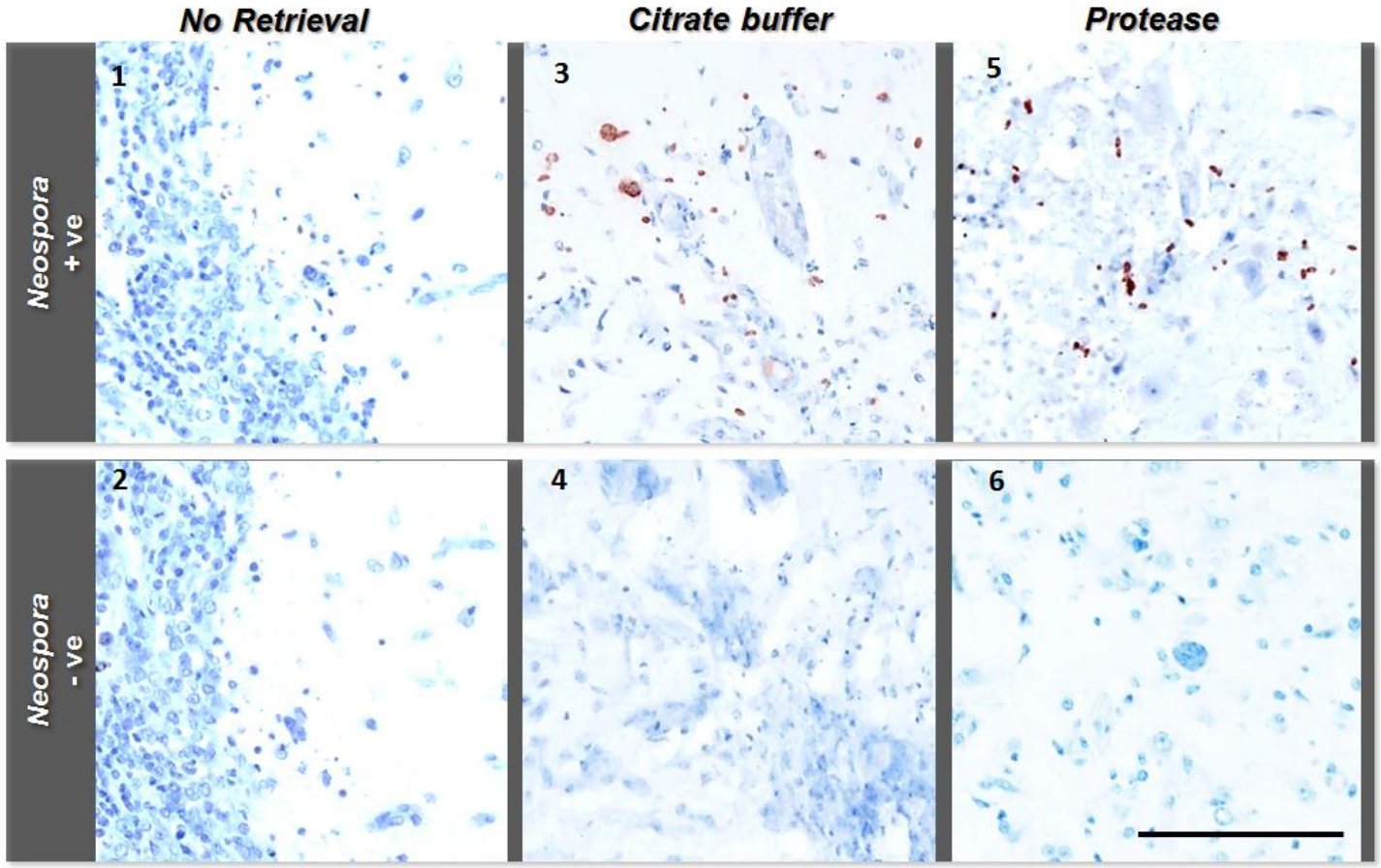


FIGURE 4. 5. IMMUNOHISTOCHEMISTRY VALIDATION OF POLYCLONAL SERUM AT A DILUTION OF 1:2000. 1-2: NO TREATMENT. 1- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT ANTI-*NEOSPORA* (NCSRS2) SERUM, 2- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT PRE-IMMUNE SERUM RA368. 3-4: HIER TREATMENT. 3- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT ANTI-*NEOSPORA* (NCSRS2) SERUM, 4- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT PRE-IMMUNE SERUM RA368. 5-6: PIER TREATMENT. 5- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT ANTI-*NEOSPORA* (NCSRS2) SERUM, 6- POSITIVE *NEOSPORA* CONTROL TISSUE TESTED WITH RABBIT PRE-IMMUNE SERUM RA368. BAR: 100µM.

4.3.2.3. Analysis of specificity of rabbit polyclonal sera using IHC

Previous in-house antisera (rabbit anti-*Neospora* -NC1, rabbit anti-*Toxoplasma*- 669-M1/M2 and rabbit anti-*Sarcocystis* 9953311), showed cross-reactive labelling for *Neospora*, *Toxoplasma* and *Sarcocystis* (**Figure 4. 6**). The results of the polyclonal rabbit sera produced from recombinant proteins (rNcSAG1, rNcSRS2 and rTgSRS2), was shown to be specific by IHC. Anti- *Neospora* (NcSAG1 and NcSRS2) specifically labelled *Neospora* parasites, but no labelling of either *Toxoplasma* or *Sarcocystis* parasites was observed (Error! Reference source not found.). Anti-*Toxoplasma* T gSRS2 specifically labelled *Toxoplasma* parasites, but no labelling was seen in *Neospora* or *Sarcocystis* control tissues (Error! Reference source not found.).

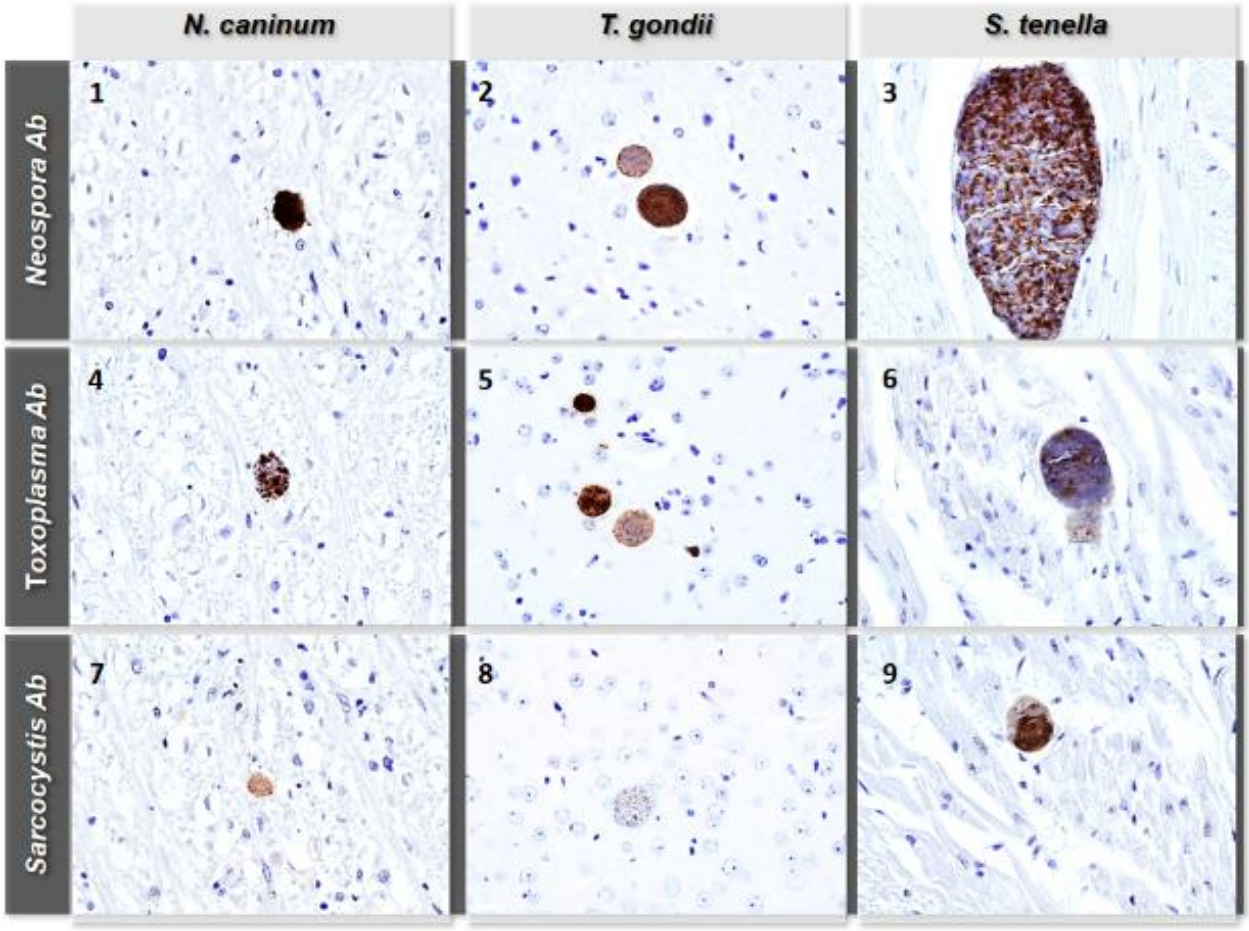


FIGURE 4. 6. IMMUNOHISTOCHEMISTRY USING IN-HOUSE RABBIT POLYCLONAL SERA. 1-3: *NEOSPORA* ANTISERA NC1 USING 1- *NEOSPORA* CONTROL TISSUE, 2- *TOXOPLASMA* CONTROL TISSUE AND 3- *SARCOCYSTIS* CONTROL TISSUE. 4-6: *TOXOPLASMA* ANTISERA (669-M1/M2) USING 4- *NEOSPORA* CONTROL TISSUE, 5- *TOXOPLASMA* CONTROL TISSUE AND 6- *SARCOCYSTIS* CONTROL TISSUE. 7-9: *SARCOCYSTIS* 9953311 USING 7- *NEOSPORA* CONTROL TISSUE, 8- *TOXOPLASMA* CONTROL TISSUE AND 9- *SARCOCYSTIS* CONTROL TISSUE. (PHOTO COURTESY OF DR GERMÁN CANTÓN, INTA, ARGENTINA).

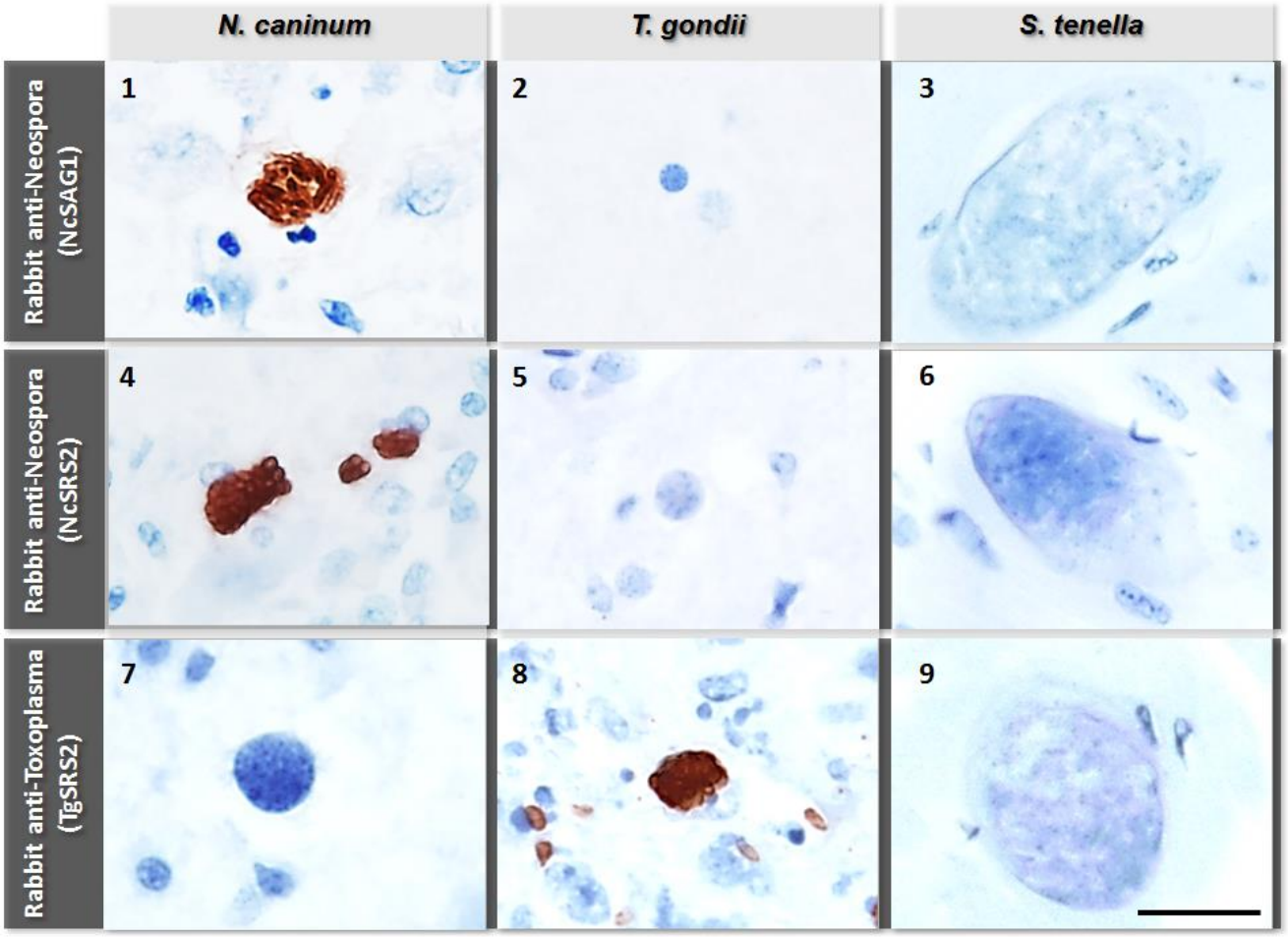
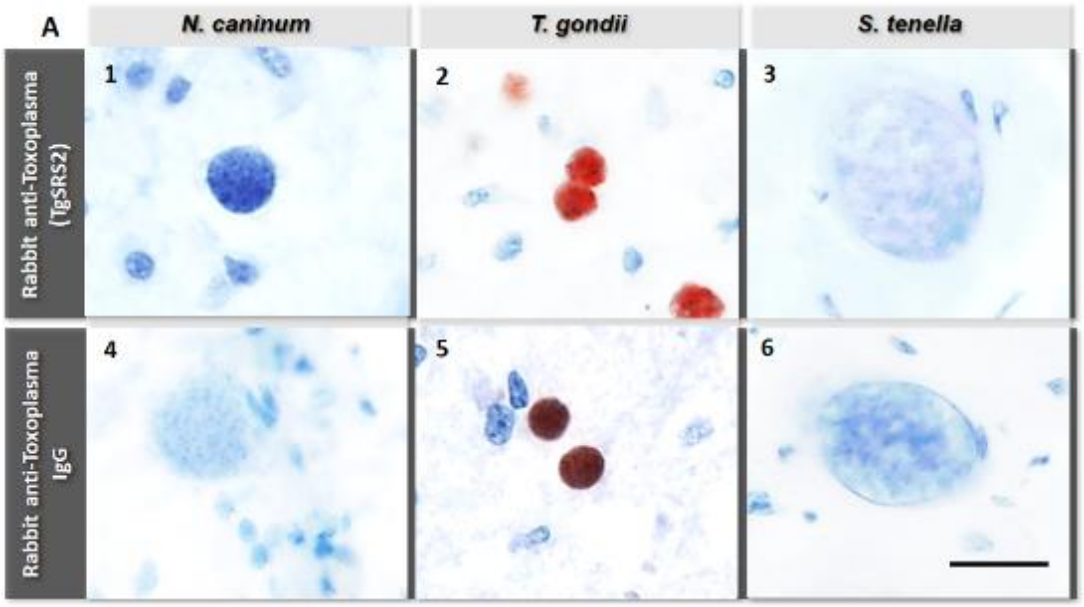


Figure 4. 7. Immunohistochemistry using polyclonal rabbit antisera. 1-3: *Neospora* antisera NcSAG1 using 1- *Neospora* control tissue, 2- *Toxoplasma* control tissue and 3- *Sarcocystis* control tissue. 4-6: *Neospora* antisera NcSRS2 using 4- *Neospora* control tissue, 5- *Toxoplasma* control tissue and 6- *Sarcocystis* control tissue. 7-9: *Toxoplasma* antisera TgSRS2 using 7- *Neospora* control tissue, 8- *Toxoplasma* control tissue and 9- *Sarcocystis* control tissue. Bar: 20µm

4.3.2.4. IHC comparison of rabbit polyclonal serum versus commercial antibodies

Results showed that both the polyclonal anti- *Toxoplasma* TgSRS2 and anti- *T. gondii* IgG were specific and only labelled *Toxoplasma* (**Figure 4. 8-A**). Neither *Neospora* or *Sarcocystis* parasites were labelled. Anti- *Neospora* NcSRS2 and NcSAG1 polyclonal sera was shown to be specific for *Neospora,* as only labelling of *Neospora* was observed. However, the commercial polyclonal goat anti- *N. caninum* showed labelling of both *Neospora* and *Toxoplasma* (**Figure 4. 8-B**). Neither polyclonal anti-*Neospora* or anti-*Toxoplasma* sera labelled *Sarcocystis* tissue. The unspecific rabbit anti- *Streptococcus agalactia* III showed labelling of all three protozoan parasites *Neospora, Toxoplasma* and *Sarcocystis* (**Figure 4. 8-C**).



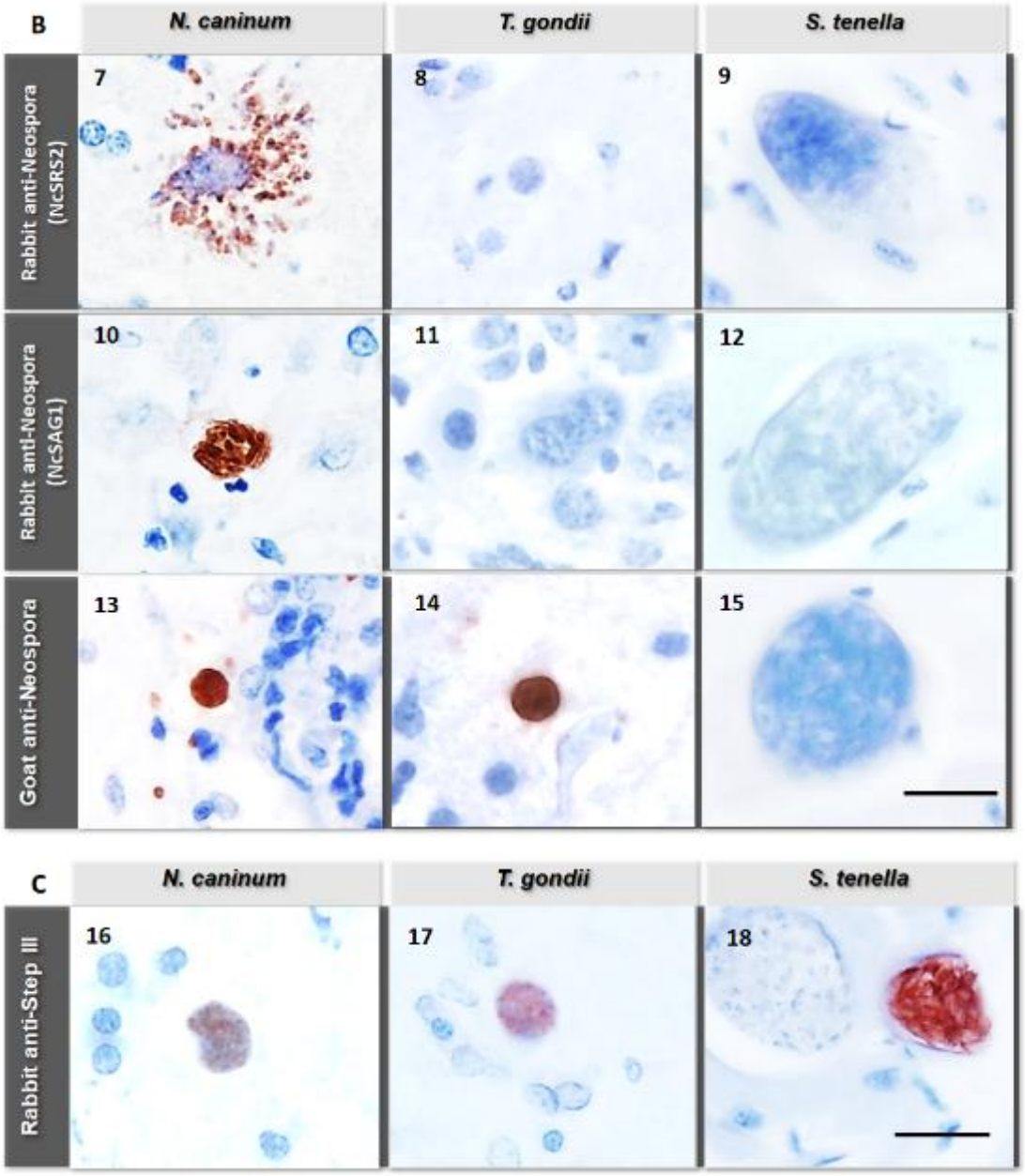


FIGURE 4. 8. IMMUNOHISTOCHEMISTRY COMPARISON OF A) 1-3- RABBIT ANTI-*TOXOPLASMA* (TGSRS2), 4-6 RABBIT ANTI- *T. GONDII* IGG ANTIBODY, B) 7-9: RABBIT ANTI- *NEOSPORA* (NCSRS2), 10-12: RABBIT ANTI- *NEOSPORA* (NCSAG1), 13-15: GOAT ANTI- *NEOSPORA* AND C*)* 16-18: RABBIT ANTI-STREPTOCOCCI AGALACTIAE III. TISSUES USED WERE *NEOSPORA* CONTROL TISSUE (1, 4, 7, 10, 13, 16), *TOXOPLASMA* CONTROL TISSUE (2, 5, 8, 11, 14, 17) AND *SARCOCYSTIS* CONTROL TISSUE (3, 6, 9, 12, 15, 18). BAR: 20µM.

4.3.2.5. Validation of antibodies using archived ruminant cases

For the validation of the polyclonal antisera 111 samples (85 bovine and 26 ovine) from foetal brain, placenta and muscle from ruminant abortion were selected, where a protozoal aetiology was suspected due to the presence of histophatological lesions. From the 111 samples tested, tissue was damaged, and results were inconclusive for 2 bovine and 1 ovine sample. Results showed that 12.0% (3/25, 95% CI: 2.55 – 31.21%) of ovine samples were positive for *T. gondii*, yet no ovine samples were positive for *N. caninum* (**Table 4. 4**). When comparing the results of the Moredun In-house antisera 41.7% (7/17) ovine samples were shown positive for *T. gondii*, of which 9/26 samples were inconclusive or not listed and no direct comparison can be made (**Appendix I Table VII**). In comparison to the PCR results a total of 56% (13/23) samples were shown positive for protozoan DNA (7 for *T. gondii*, 4 for *S. gigantea*, 2 for *S. tenella* DNA), of which 3/26 samples were not tested (**Appendix I Table VII**). Moreover, results of all samples showed positive labelling of 1.9% (2/108, 95% CI: 0.23 - 6.53%) with the anti-*Toxoplasma* TgSRS2 serum (**Table 4. 4**). Both cases shown positive with antiserum *Toxoplasma* TgSRS2 for *T. gondii*, were shown positive for *T. gondii* by PCR and previous Moredun In-house antisera (**Appendix I Table VII**). Results showed positive labelling of 2.8% (3/108, 95% CI: 0.58 – 7.90%) with the anti- *T. gondii* IgG antibody (**Table 4. 4 and Appendix I Table VII**), of which 2 were shown positive for *T. gondii* by PCR and positive using the old *Toxoplasma* antisera. One case was shown positive with anti- *T. gondii* IgG antibody was previously negative using the old *Toxoplasma* antisera (**Table 4. 4 and Appendix I Table VII**).

For bovine samples 27.7% (23/83, 95% CI: 1.84 – 38.62%) were positive for *N. caninum*, yet no bovine samples were positive for *T. gondii* (**Table 4. 4**). When comparing the results of the Moredun In-house antisera 47.4% (36/76) of bovine samples were shown positive for *N. caninum*, of which 9/85 samples were inconclusive or not listed and no direct comparison can be made (**Appendix I Table VII**). In comparison to the PCR results, a total of 28.6% (16/56) of bovine samples were shown positive for protozoan DNA (13 for *N. caninum*, 2 for *T. gondii* and 1 for *S. cruzi* DNA), of which 29/85 samples were not tested (**Appendix I Table VII**). Moreover, results showed that for the anti- *Neospora* NcSAG1 serum, 14.8% (16/108, 95% CI:

8.71 – 22.94%) of all ruminant (15 bovine and 1 ovine) samples were positive for *Neospora* (**Table 4. 4 and Appendix I Table VII**). Of those 16 positive samples with antiserum *Neospora* NcSAG1, 9 samples were previously positive for *N. caninum* using the Moredun in-house antisera and 7 samples were negative (**Appendix I Table VII**). Four cases positive with *Neospora* NcSAG1, were shown positive for *Neospora* (n=3) and *Toxoplasma* (n=1) by PCR (**Appendix I Table VII**). Twenty-four blocks previously positive using the old *Neospora* antiserum were shown negative using the anti- *Neospora* NcSAG1 serum (**Appendix I Table VII**). For the anti- *Neospora* NcSRS2 serum, 21.3% (23/108, 95% CI: 14.00 – 30.22%) of ruminant samples showed positive labelling for *Neospora*. Of those samples shown positive with antiserum *Neospora* NcSRS2, 15 of those cases were previously positive and 8 were shown negative for *Neospora* using the old *Neospora* antiserum (**Appendix I Table VII**). Seven cases positive for *Neospora* NcSRS2 were confirmed positive for *Neospora* by PCR (**Appendix I Table VII**). Twenty-one blocks previously tested positive using the old *Neospora* antiserum were shown negative using the anti-*Neospora* NcSRS2 serum (**Appendix I Table VII**).

The statistical analysis revealed that there is no significant difference of labelling (P= 0.388) between anti-*Neospora* NcSRS2 and rabbit anti- *Neospora* NcSAG1. Yet, results showed that 6 cases were only positive with anti-*Neospora* NcSRS2 and not with anti- *Neospora* NcSAG1. No cases where positive for only anti- *Neospora* NcSAG1 and negative for anti-*Neospora* NcSRS2. No significant difference (P= 1.000) between labelling by anti-*Toxoplasma* TgSRS2 and anti-*T. gondii* IgG antibody was observed. No labelling of protozoan parasites was observed in 85.2%, 78.7%, 98.1% and 97.2% of all ruminant samples for anti-NcSAG1, anti-NcSRS2, anti-TgSRS2 and anti-*T. gondii* IgG antibody, respectively (**Appendix I Table VII**). No samples were shown positive for both *Neospora* and *Toxoplasma*.

In 6.5% (7/108, 95% CI: 2.64 - 12.89%) of sample tissues, no labelling was observed, yet structures were identified that were similar and compatible with sarcocysts (**Appendix I Table VII**). PCR results confirmed that those 7 samples were idenfied positive for *Sarcocystis* species and showed that 4 samples were positive for *S. gigantea*, 2 for *S. tenella* and 1 for *S. cruzi* (**Appendix I Table VII**). Previous IHC

analysis using the Moredun In-house antisera (anti- Sarco) showed labelling for *Sarcocystis* for 4 samples.

TABLE 4. 4. IHC SUMMARY RESULTS OF POSITIVE LABELLING OF PROTOZOAN PARASITES USING NCSAG1, NCSRS2, TGSRS2 AND RABBIT ANTI-*T. GONDII* IGG ANTIBODY (THERMOFISHER).

	Positive (%) (n°/total)		Negative (%) (n°/total)	
Antiserum/ Antibody	**Ovine**	**Bovine**	**Ovine**	**Bovine**
rabbit anti-*Neospora* (NcSAG1)	0 (0/25)	19 (16/83)	100 (25/25)	80.7 (67/83)
rabbit anti-*Neospora*, (NcSRS2)	0 (0/25)	27.7 (23/83)	101 (25/25)	72.3 (60/83)
rabbit anti-*Toxoplasma* (TgSRS2)	7.7 (2/25)	0 (0/83)	92.3 (23/25)	100 (83/83)
anti-*T. gondii* IgG antibody (Thermofisher)	12.0 (3/25)	0 (0/83)	88.0 (22/25)	101 (83/83)

4.3.5. Screening of monoclonal antibodies

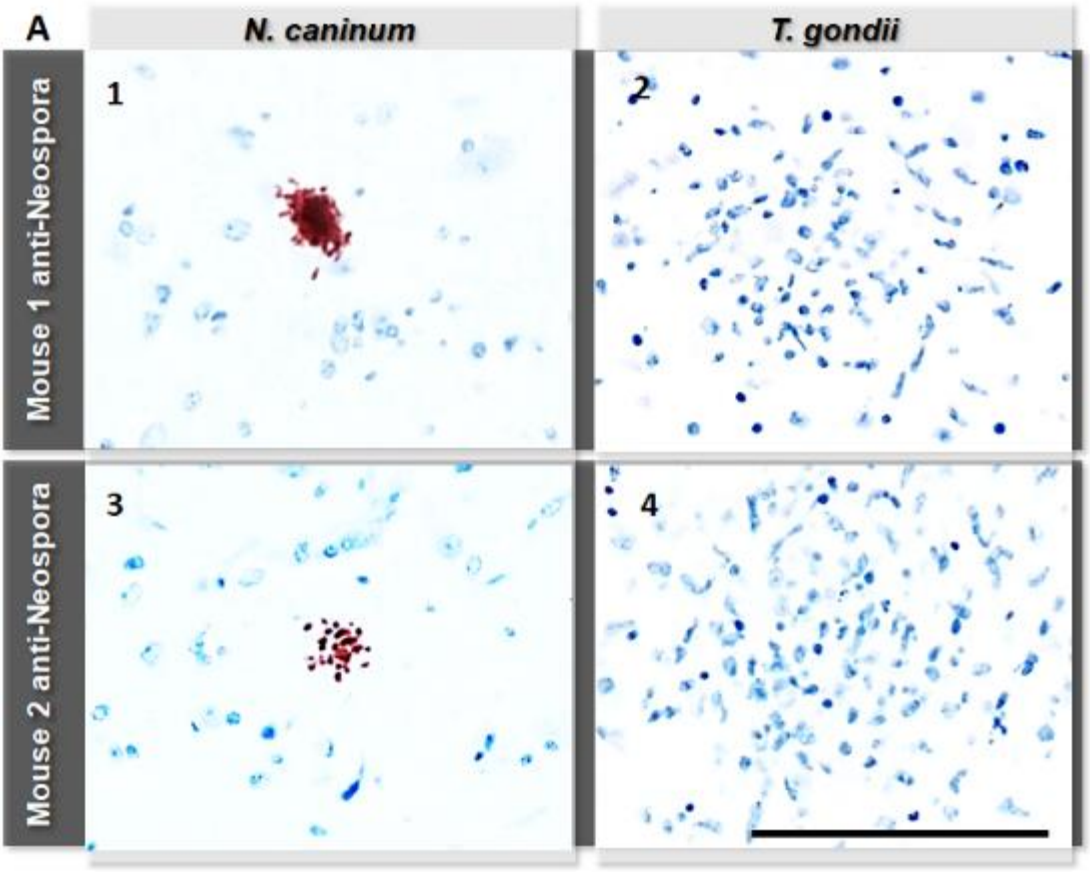
4.3.5.1. Verification of functionality and specificity of mouse sera

ELISA results revealed that all four mice immunised with *Neospora* recombinant protein (rNcSRS2) showed reactivity against the *Neospora* antigen fraction (**Table 4. 5)**. No reactivity was observed with *Toxoplasma* antigen fraction, and all four were used in IHC testing. All mice immunised with *Toxoplasma* recombinant protein (rTgSRS2) showed reactivity against the *Toxoplasma* antigen fraction. However, mice 3 and 4 showed cross-reactivity against the *Neospora* antigen fraction (**Table 4. 5**). Only mice 1 and 2 were continued for IHC analysis.

TABLE 4. 5. ELISA AND IHC RESULTS SHOWING REACTIVITY AND SPECIFICITY OF MICE USED FOR POLYCLONAL SERA PRODUCTION. +VE INDICATED A POSITIVE REACTION AND -VE INDICATES A NEGATIVE REACTION TOWARDS *NEOSPORA* / *TOXOPLASMA*. ND- NOT DONE.

Tests	**ELISA**		**IHC**	
	***Neospora* antigen**	***Toxoplasma* antigen**	***Neospora***	***Toxoplasma***
	Neospora polyclonal sera			
Mouse 1	+	-	+	-
Mouse 2	+	-	+	-
Mouse 3	+	-	+	-
Mouse 4	+	-	+	-
	Toxoplasma polyclonal sera			
Mouse 1	-	+	-	+
Mouse 2	-	+	-	+
Mouse 3	+	+	ND	ND
Mouse 4	+	+	ND	ND

IHC results revealed that *Neospora* mouse 1, 2, 3 and 4 sera showed strong labelling of *Neospora* parasites and no labelling of *Toxoplasma* was observed (**Figure 4. 9)**. *Toxoplasma* immunised mice 1 and 2 sera showed strong labelling of *Toxoplasma* parasites and no labelling of *Neospora* parasites was observed (**Figure 4. 9**). No labelling was observed for the negative controls using normal mouse serum. Based on the ELISA and IHC results, mice 1 and 2 were used for hybridoma clone production for *Neospora* and *Toxoplasma*.



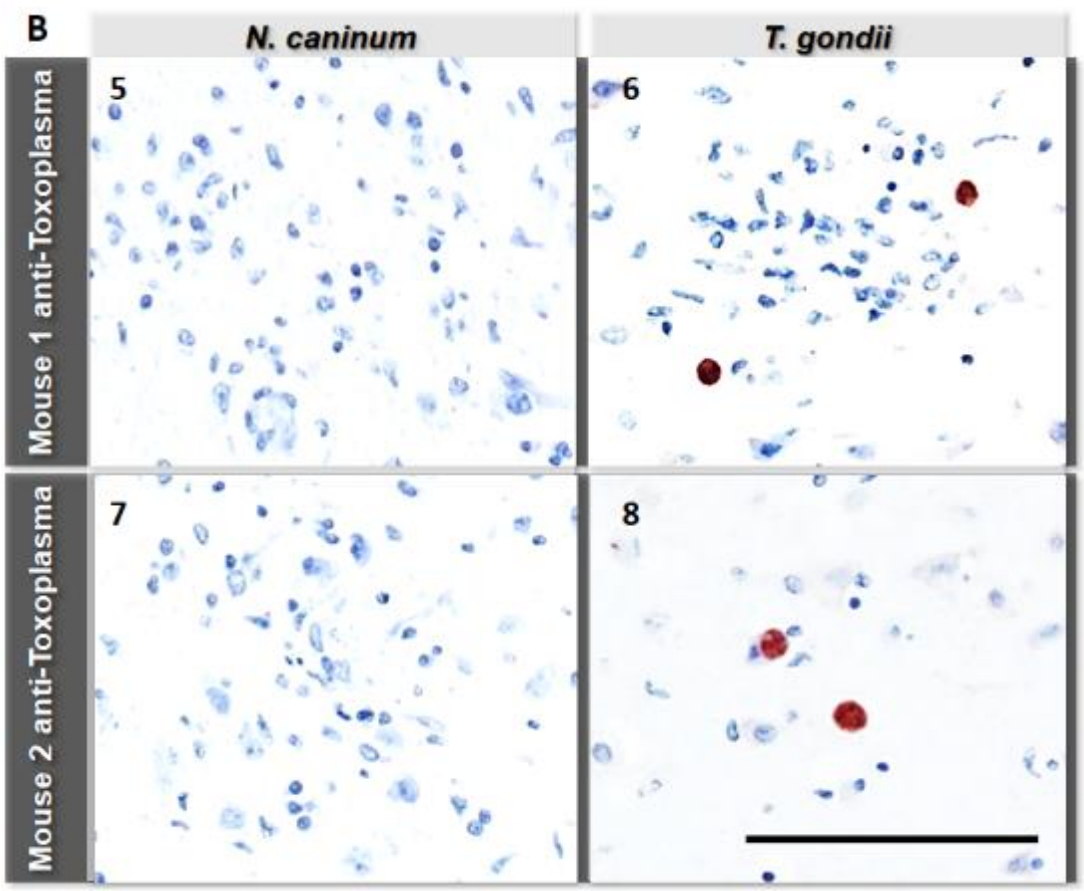


FIGURE 4. 9. IMMUNOHISTOCHEMISTRY TESTING FUNCTIONALITY AND SPECIFICITY OF POLYCLONAL MOUSE SERUM. A) 1-2 MOUSE 1 ANTI-*NEOSPORA*, 3-4 MOUSE 2 ANTI-*NEOSPORA*. B) 5-6 MOUSE 1 ANTI-*TOXOPLASMA*, 7-8 MOUSE 2 ANTI-*TOXOPLASMA* USING *NEOSPORA* CONTROL TISSUE (1, 3, 5, 7) AND *TOXOPLASMA* TISSUE (2, 4, 6, 8). BAR: 100µM.

4.3.5.3. Verification of functionality and specificity of hybridoma clones

A total of 2350 hybridoma lines were produced for each *Neospora* and *Toxoplasma*. Of the 2350 hybridomas, 55 for *Neospora* showed reactivity against *Neospora* antigen. Five hybridomas showed cross-reactivity against *Toxoplasma* antigen and were discontinued (**Table 4. 6**). Following further incubation, ELISA results showed that only 37 parental hybridomas remained reactive with an OD of over 0.25 with *Neospora* antigen and 13 showed no reactivity (**Table 4. 6**). The hybridomas that showed the highest reactivity with ELISA were tested using IHC.

TABLE 4. 6. ELISA RESULTS OF PARENTAL HYBRIDOMA LINES FROM MICE IMMUNISED WITH *NEOSPORA* AND *TOXOPLASMA*.

Total parental hybridomas	Hybridoma reactivity				Cross-reactivity	Taken forward
	OD 1-9.999	OD 0.5-0.999	OD 0.25-0.499	No reactivity		
Neospora	*Neospora* antigen				*Toxoplasma* antigen	
55	25	7	5	13	5	37
Toxoplasma	*Toxoplasma* antigen				*Neospora* antigen	
25	13	2	3	4	3	18

For *Neospora*, 12 out of 37 hybridomas, supernatants were tested using IHC and results revealed that three parental hybridomas were suitable for limited dilution cloning. IHC results showed that two parental *Neospora* hybridomas showed faint labelling for *Neospora* and one showed strong labelling (**Figure 4. 10**). All 3 *Neospora* hybridomas (ME7, GF7, AB1) showed specificity to *Neospora* (**Figure 4. 10)**, and no labelling for *Toxoplasma* was observed.

Of the 2350 hybridoma lines, 25 showed reactivity against *Toxoplasma* antigen. Three hybridomas showed reactivity against *Neospora* antigen and were discontinued. Following further incubation, ELISA results showed that only 18 hybridomas remained reactive against *Toxoplasma* antigen with an OD of over 0.25, and 4 showed no reactivity (**Table 4. 6**). For *Toxoplasma*, all 18 hybridoma supernatants were tested using IHC, and results revealed that two (LF11 and FD11) were suitable for limiting dilution cloning. Both *Toxoplasma* hybridomas showed labelling for *Toxoplasma* parasites, but not for *Neospora* (**Figure 4. 10)**. However, following limiting dilution cloning, ELISA results revealed that LF11 and FD11 were no longer positive against the *Toxoplasma* antigen (Data not shown). No *Toxoplasma* monoclonal antibody was generated.

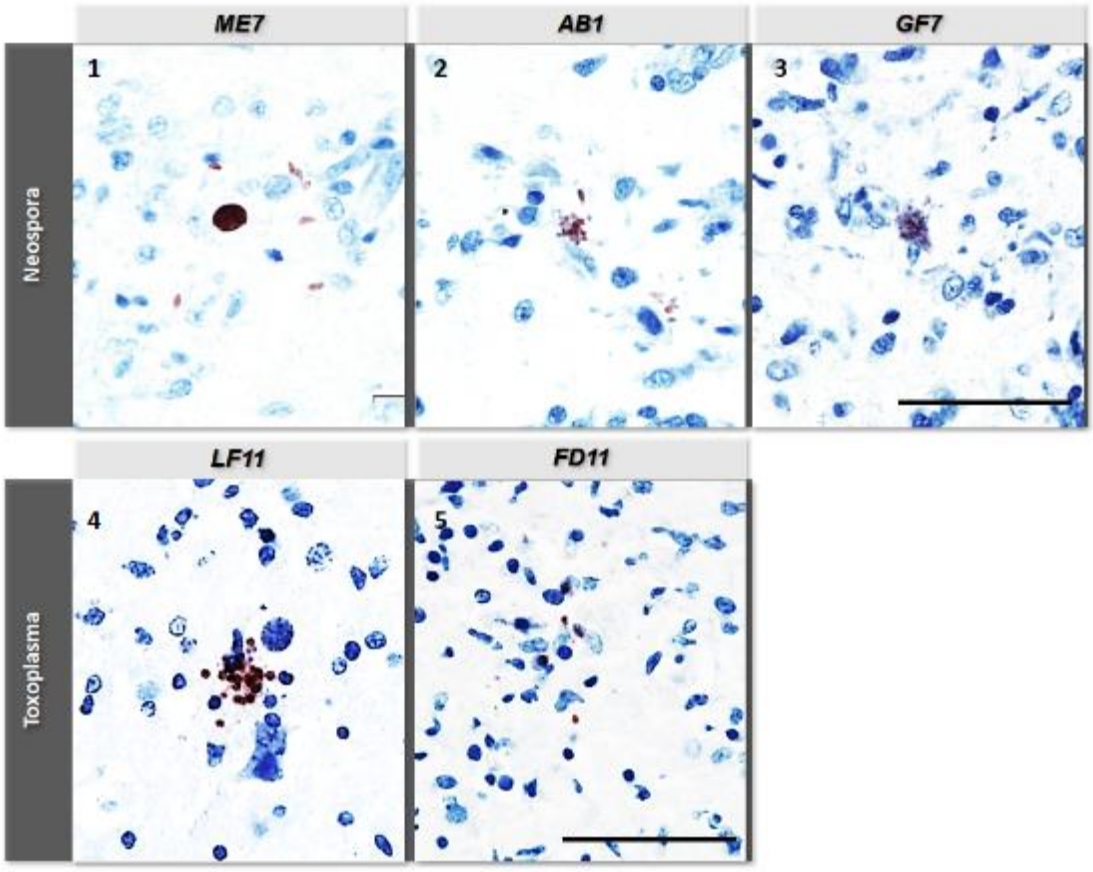


FIGURE 4. 10. IMMUNOHISTOCHEMISTRY TESTING FUNCTIONALITY OF THE 1-3 *NEOSPORA* PARENTAL HYBRIDOMA (ME7, AB1, GF7) USING *NEOSPORA* CONTROL TISSUE AND 4-5 *TOXOPLASMA* PARENTAL HYBRIDOMA (LF11 AND FD11) USING *TOXOPLASMA* CONTROL TISSUE. BAR: 50µM.

Following limited dilution cloning of *Neospora* hybridomas, ELISA results revealed that only sub-clones ME7 and GF7 remained positive with *Neospora* antigen. IHC analysis revealed that ME7 sub-clones (ME7- B12, ME7- D11 and ME7- A10) showed labelling of *Neospora* parasites and no labelling with *Toxoplasma* parasites (**Figure 4. 11**). However, results for GF7 sub-clones (GF7- E8, GF7- G7, and GF7- H10) showed no labelling with either *Neospora* or *Toxoplasma* parasites.

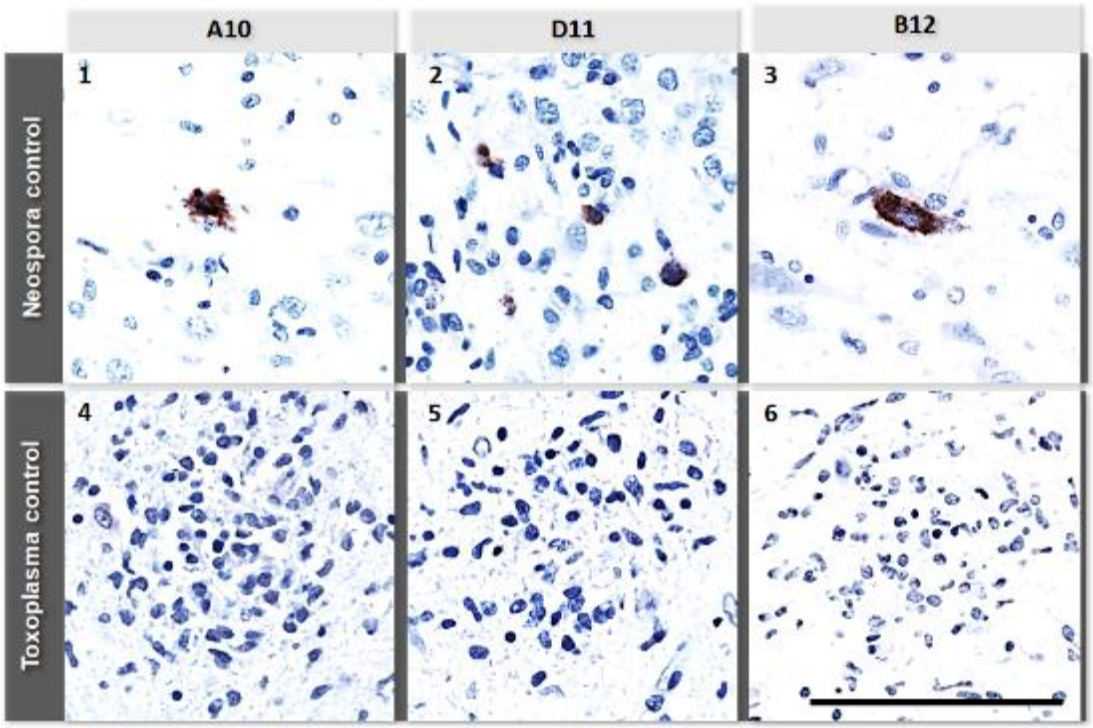


FIGURE 4. 11. IMMUNOHISTOCHEMISTRY FUNCTIONALITY TESTING OF THE *NEOSPORA* ME7 HYBRIDOMA SUB-CLONES 1,4) A10, 2,5) D11, AND 3,6) B12 AGAINST *NEOSPORA* CONTROL BLOCKS (1-3) AND *TOXOPLASMA* CONTROL BLOCK (4-6). BAR: 100µM.

The ME7 *sub-clone* B12 was further re-cloned, and ME7.1.B12.C9. supernatant was collected containing the monoclonal antibody. Results showed labelling of *Neospora* parasites using 20 µg/ml and 5 µg/ml and 2 µg/ml (**Figure 4.** 12). However, using 20 µg/ml and 5 µg/ml showed additional labelling using the negative control and IHC examination revealed that labelling using the monoclonal antibody worked best at a dilution of 2 µg/ml. Following dilution, the *Neospora* monoclonal antibody (ME7.1.B12.C9) was tested for specificity. Results showed that the monoclonal antibody ME7.1.B12.C9 was specific and only labelling of *Neospora* (**Figure 4. 13**). No labelling of *Toxoplasma* or *Sarcocystis* parasites were observed (**Figure 4. 13**).

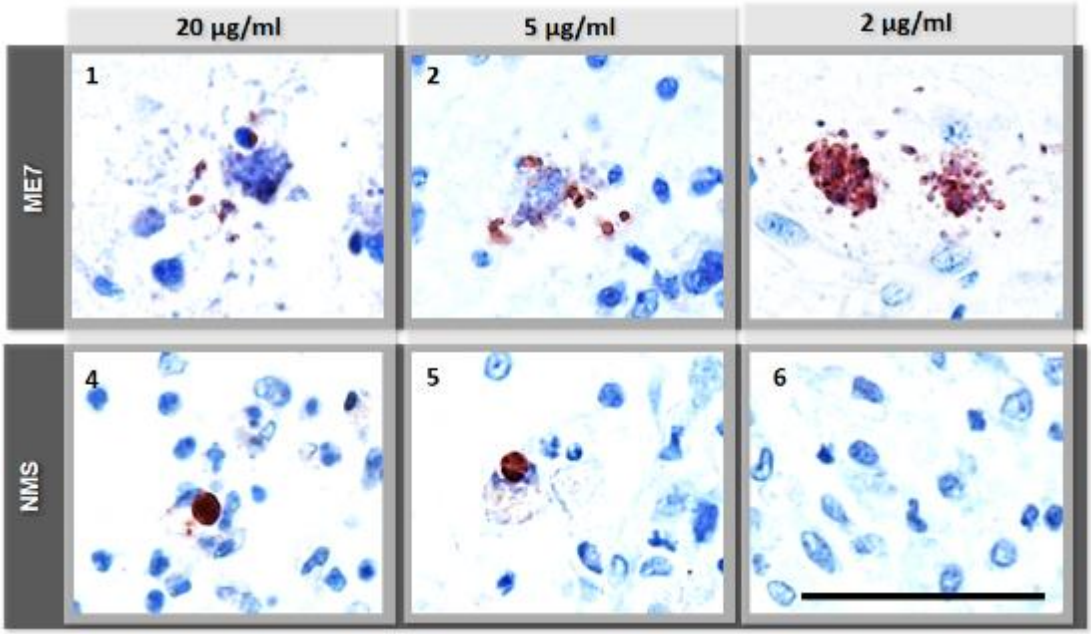


FIGURE 4. 12. IHC DILUTION ANALYSIS OF 1) *NEOSPORA* ME7 ANTIBODY CONCENTRATION 20 µG/ML, 2) *NEOSPORA* ME7 ANTIBODY CONCENTRATION 5µG/ML, 3) *NEOSPORA* ME7 ANTIBODY CONCENTRATION 2 µG/ML, 4) NORMAL MOUSE SERUM CONCENTRATION 20 µG/ML, 5) NORMAL MOUSE SERUM CONCENTRATION 5µG/ML, 6) NORMAL MOUSE SERUM CONCENTRATION 5µG/ML. BAR 50 µM.

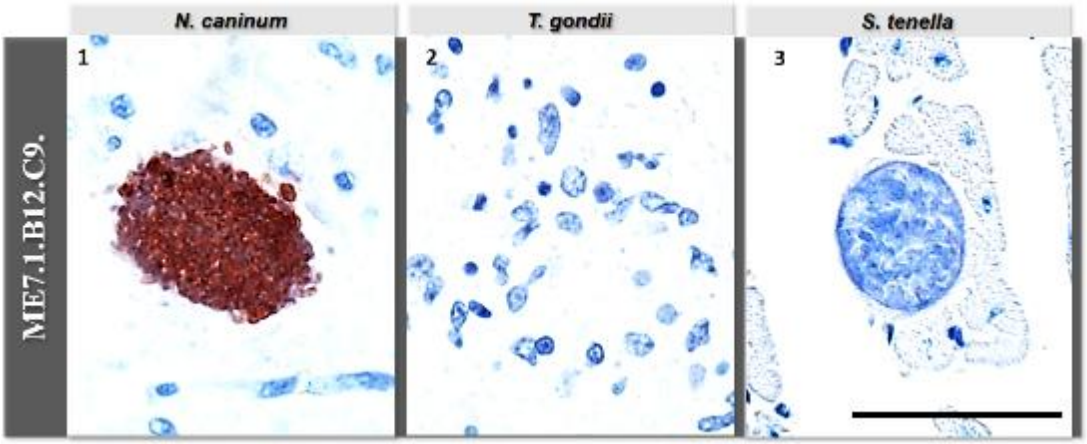


FIGURE 4. 13. IHC SPECIFICITY TESTING OF *NEOSPORA* MONOCLONAL ANTIBODY ME7.1.B12.C9. 1) *NEOSPORA* CONTROL TISSUE, 2) *TOXOPLASMA* CONTROL TISSUE AND 3) *SARCOCYSTIS* CONTROL TISSUE. BAR: 50µM

Chapter 4.4. Discussion

This chapter discussed the development of rabbit polyclonal antiserum, and the production of monoclonal antibodies raised against recombinant proteins generated in Chapter 3.

4.4.1. Polyclonal antibody project

Initially, rabbits were chosen for the production of polyclonal antibodies due to cost and location convenience. Rabbits are capable of producing a high-titre antibody response, commonly generating around 5-10 mg/ml rabbit antiserum of from 20ml of serum (Burns *et al.*, 2005; Dako, 2013; Stills, 2012). Polyclonal antibodies are more sensitive yet less specific than monoclonal antibodies, and have shown cross-reactivity amongst closely related parasites, such as *Neospora*, *Toxoplasma* and *Sarcocystis* (Ramos-Vara *et al.*, 2008). Many studies have reported cross-reactivity between *Neospora* and *Toxoplasma* using IHC or serological analysis when whole parasite antigens were used (Dubey *et al.*, 1996; Gondim *et al.*, 2017; McAllister *et al.*, 1996b; Peters *et al.*, 2000; Silva *et al.*, 2007; Sundermann *et al.*, 1997; Van Maanen *et al.*, 2004). As multiple epitopes are recognised by rabbit polyclonal serum, the potential for cross-reactivity is greatly increased. Additionally, using closely related parasites, such as *Toxoplasma* and *Neospora* could increase the potential for cross-reactivity, as they share similar structural proteins and functions.

Most diagnostic strategies using antibodies are based on the detection of antigens from whole parasite lysates or fixed tachyzoites (Dubey & Schares, 2011; Sa *et al.*, 2014). Similar results were also observed in our study, with antiserum raised against whole parasite lysates which displayed cross-reactivity between *Neospora*, *Toxoplasma* and *Sarcocystis* spp. (section 4.3.2.3). Using a single antigen, such as a parasite surface protein (i.e. SAG or SRS) could provide an alternative method for producing specific antibodies for diagnostic tests (Burns *et al.*, 2005; Zhang *et al.*, 2011). Genus-specific antibodies were thus raised against recombinant proteins, targeting the unique regions of surface antigens (SAG and SRS) to enable a specific diagnosis of the parasites in ruminant abortion cases using IHC.

The polyclonal antisera were produced using New Zealand white rabbits, and the production was initiated with a screening of 14 rabbit pre-immune sera. Screening of the pre-immune serum was performed to indicate which animal was most likely to give the lowest background level of binding, and eliminate factors that could potentially lead to cross-reactivity of antibodies with the protozoal parasites in downstream applications (Abcam; Leenaars *et al.*, 1999). Moreover, testing individual rabbit pre-immune sera shows how the animal will respond to the immunogen, and most importantly what antibodies are already present in their system (Abcam; Lathrop *et al.*, 2006). The results of the pre-immune screening revealed that only a few rabbits were suitable for antibody production, as the rabbit sera showed reactivity with *Neospora*, *Toxoplasma* and *Sarcocystis*, as well as giving a high amount of unspecific background staining. Pre-screening of rabbit sera is an important test to determine the suitability of rabbits, especially for the production of specific antibodies. Our results have demonstrated the difficulty of finding suitable rabbits for the production of anti-sera against protozoan parasites, as unspecific antibodies already in their system were able to recognize protozoan parasites.

Rabbits are known to produce large amounts of non-specific antibodies, creating high background signals during IHC analysis (Lathrop *et al.*, 2006). Many environmental factors can encourage immune responses prior to the immunisation procedure against various pathogens (i.e. bacteria, fungi, and viruses) (Lathrop *et al.*, 2006; Leenaars *et al.*, 1999). However, no reports have investigated reactivity of the pre-immune sera with the protozoans *Neospora*, *Toxoplasma* or *Sarcocystis* prior to immunisation, which suggests that some cross-reactive epitopes in the environment can trigger an immune response in rabbits against protozoan parasites. Similar results were seen in a study by Lathrop *et al.* (2006), where 19 different rabbit pre-immune sera were tested against 27 pathogenic and non-pathogenic bacterial cultures using ELISA analysis. The results showed that 53% of the rabbit pre-immune serum showed positive reactions with 10 or more bacterial cultures (Lathrop *et al.*, 2006). These and our results suggest that 'the presence of cross-reactive antibodies in the pre-immune serum' against microorganisms, such as bacteria and protozoa, is a common problem when using rabbits to develop specific antisera (Lathrop *et al.*, 2006).

From our pre-immunisation screening results, only 3 rabbits were chosen. The rabbit that showed reactivity with *Toxoplasma* was immunised with the *Toxoplasma* recombinant protein, and the rabbit showing reactivity with *Neospora* was immunised with *Neospora* recombinant protein to avoid cross-reactivity at a later stage. Immunisation with a *Toxoplasma* recombinant protein with a rabbit that shows reactivity towards *Neospora* could potentially enhance non-specific antibodies, as an increase in reactivity between protozoan parasites and the non-relevant antisera (Rabbit anti- *S. agalactia III*) was observed.

Each rabbit was immunised against the recombinant protein selected using Freund's adjuvant. Using adjuvants has shown to lower the amount of antigen required and prolongs the antigen stimulation of the host immune system (Burns *et al.*, 2005; Ebersbach *et al.*, 2016). Moreover, Freund's adjuvant has shown to contain heat-inactivated bacterial elements that enhance the immune response by directly stimulating the activity of the antigen-presenting cells (Billiau & Matthys, 2001; Ebersbach *et al.*, 2016).

The western blot analysis identified the functionality, specificity and cross-reactivity of the polyclonal sera towards *Neospora* / *Toxoplasma* antigens and recombinant proteins. Both anti-*Neospora* sera showed a strong reactivity against recombinant proteins (rNcSRS2 and rNcSAG1) and *Neospora* antigen fractions, suggesting that the polyclonal serum can react with other potential surface proteins of the same species. The lack of reactivity against the *Toxoplasma* antigen fraction and recombinant protein suggests that the anti- *Neospora* sera specifically recognise *Neospora*, and could be used during IHC analysis. Similar results were observed in the study by Nishikawa *et al.*, (2002), who reported no cross-reactivity of the recombinant surface antigen (NcSRS2) with anti-*T. gondii* cat or mouse sera.

Similar results were observed with the anti-*Toxoplasma* sera, which was shown to be specific as no cross-reactivity with *Neospora* antigen or recombinant proteins were observed, indicating that this polyclonal serum is specific for *Toxoplasma*. However, results showed a lack of reactivity of the anti-*Toxoplasma* serum against the *Toxoplasma* antigen fractions (towards either the water-soluble parasite fraction or the

parasite pellet). This could be because the specific target epitope was not displayed during the antisera development, potentially as a result of refolding of the recombinant protein, and hence the *Toxoplasma* antigen fraction was not recognised by the *Toxoplasma* polyclonal serum. Studies have shown that it is important that proteins used for immunisation are homogenous, correctly folded and presented such that the critical epitopes are accessible (Ebersbach *et al.*, 2016). Each recombinant protein expressed was insoluble, and even though refolding procedures were used, some proteins are not able to reproduce the properties of the native protein (Dong *et al.*, 2012). Folding of proteins could be assessed by activity assays, such as SDS-PAGE gels, size exclusion chromatography, reversed-phased HPLC assay or differential scanning fluorimetry guided protein refolding assay, which could allow discrimination between folded, misfolded and unfolded states of refolded proteins (Wang *et al*., 2017). 'Protein oxidation, aggregation and degradation are known issues which can affect the outcome of antibody generation severely', hence why it was important to test functionality and specificity of the polyclonal sera using several assays, i.e. western blots and IHC (Ebersbach *et al.*, 2016). The reactivity and specificity of the polyclonal serum should be tested using the appropriate antigen (i.e. recombinant proteins and parasite antigens). Having immunoreactivity of the polyclonal sera in one assay (i.e. western blot) does not mean that the anti-sera will show reactivity and specificity using a different assay (IHC) (Ramos-Vara *et al.*, 2008).

IHC was performed to evaluate the functionality and specificity of the polyclonal sera on fixed tissue samples containing *Neospora*, *Toxoplasma* and *Sarcocystis* parasites. The standardization of the anti-sera is necessary in order to derive the optimum working conditions that ensure the highest specificity and labelling with the least amount of background. Studies have shown that binding of the antibody to the antigen can be influenced by various factors, such as concentration of antigen, temperature, buffer, pH, as well as pre-analytical, analytical and post-analytical factors (Bordeaux *et al.*, 2010; Hardy *et al.*, 2013a). Thus determining the relative and optimum concentrations of an antibody can influence the antibody-antigen complex formation for optimal results (Bordeaux *et al.*, 2010; Hardy *et al.*, 2013a). An optimum dilution for each polyclonal serum was obtained.

Most tissue specimens for IHC analysis are fixed in formalin and embedded in paraffin wax, as this procedure preserves the morphology and structure of the tissues. The fixation process causes the proteins to cross-link, which may result in a loss of the ability of the antibodies to bind to the antigens, as the epitopes are masked (Abcam; Dako, 2013; Gown, 2004; Shi & Taylor, 2013). Retrieval methods are meant to break the methylene bridges, allowing exposure of antigenic sites that were previously masked. Masked epitopes can be retrieved or 'unmasked' using antigen retrieval methods, such as PIER or HIER methods, and the correct retrieval treatment needs to be established to obtain optimum working conditions. In the study by Ramos-Vara & Beissenherz (2000), the authors tested different retrieval methods on different antibodies, showing that 84% of the 63 antibodies tested needed some type of antigen retrieval for optimal results. They showed that heat retrieval was the preferred method for 49.2% of antibodies compared to enzyme digestion for 35% of antibodies (Ramos-Vara & Beissenherz, 2000).

In our study, using the heat retrieval (HIER) method resulted in better labelling of the parasites compared to not using a retrieval treatment. However, HIER altered the morphology of the tissues sample tested, making the results obtained more difficult to be interpreted. Indeed, various studies have shown that when using HIER, tissues are essentially boiled/cooked. The action of the high temperature on the formaldehyde-fixed proteins produces hydrolysis, which can cause the cross-links to break down and damage the morphology of the specimen (Abcam; Gown, 2004; Shi & Taylor, 2013). Additionally, boiling the material can cause the tissue to be released off the slide, and can potentially lead to an inadequate processing of the sample (Shi & Taylor, 2013).

From our study, the PIER treatment resulted in much stronger labelling intensity and clearer tissue morphology compared to the HIER treatment, and was selected as the preferred method. The study by Battifora and Kopinski (1986), showed that PIER is the preferred method if the tissues are fixed for long periods in formaldehyde, and hence require exposure to a proteolytic enzyme. For any antibody produced, various retrieval method should be tested to achieve optimum results (Ramos-Vara & Beissenherz, 2000). In the present study, once optimal conditions were achieved, each polyclonal serum was tested for specificity using tissue blocks from cases known to

be experimentally or naturally infected with *Neospora* / *Toxoplasma* / *Sarcocystis*. The IHC results indicated that each rabbit polyclonal serum raised against recombinant proteins was specific, as no cross-labelling with other protozoan parasites was observed. Various studies have demonstrated similar results using other serological techniques, such as ELISA, and have shown that when recombinant proteins were used to raise antibodies, the resulting antisera did not results in cross-reactivity between *Neospora* and *Toxoplasma* (Dong *et al.,* 2012; Nishikawa *et al.*, 2002; Zhang *et al.*, 2011).

Species-specific proteins, such as surface antigens, were previously identified as potential diagnostic markers (Kotresha & Noordin, 2010; Zhang *et al.*, 2011). In the study by Dong *et al.*, (2012), recombinant proteins (rNcSRS2, rNcSAG1 and rNcGRA7) were recognised by *Neospora*-specific antibodies in cattle serum, and resulted in reliable and specific detection of *Neospora* infection using ELISA techniques. During the present study study, the polyclonal sera were compared with commercially available antibodies that were raised against whole parasite lysates. Results revealed that the commercial polyclonal anti-*Neospora* serum (VMRD), was unspecific, as it labelled both *Neospora* and *Toxoplasma* parasites. This suggested that sera raised against whole parasite lysates has a greater cross-reactive potential compared to sera raised against specific recombinant proteins. Similar findings using ELISA analysis had reported cross-reactivity between *Neospora* and *Toxoplasma* when whole parasite lysates were used (Chahan *et al.*, 2003).

The results using the commercially available anti- *T. gondii* IgG antibody showed that it was specific as no labelling of *Neospora* or *Sarcocystis* was observed, even though it was raised against a whole parasite lysate. An explanation could be that the commercial rabbit antisera was purified, eliminating the binding to non-specific epitopes. Purification of an antiserum involves the selective enrichment or specific isolation of an antibody from the serum, as antiserum can contain both target and non-target antibodies (Abcam; Dako, 2013). The specific-target isotype ‘IgG’, which was purified from antiserum could be more specific than using the whole antiserum. Even though purification of the polyclonal serum enables elimination of serum proteins and non-specific antibodies and enriches fractions of the immunoglobulin for specific

epitopes (i.e. IgG fraction), it could potentially result in a loss of affinity of other potential specific immunoglobulin fractions and a loss of sensitivity towards the target antigen. Moreover, this commercial polyclonal antibody is no longer available.

Lastly in this study, the non-relevant polyclonal anti-Strep.III serum showed labelling of all protozoan (*Toxoplasma*, *Neospora* and *Sarcocystis).* Although a non-related protein (*S. agalactia* III protein) to the target epitope was used to immunise the rabbits, strong labelling of *Toxoplasma*, *Neospora* and *Sarcocystis* was observed, showing the potential pre-existence of cross-reactive antibodies against protozoal parasites in the rabbit's immune serum. The presence of non-specific antibodies in the rabbit serum have previously not shown to be taken into consideration and very few studies have tested the rabbit status. However, our study has demonstrated that testing pre-immune sera is crucial for the production of polyclonal antibodies as it highlights the presence of pre-exiting antibodies against protozoan parasites.

Further evaluation of each polyclonal serum was performed using ruminant (sheep and cattle) cases, and results demonstrated that each polyclonal antisera was able to identify and detect specific protozoan parasites in the tested tissues. The *Neospora* antisera enabled diagnosis of 14.8% and 21.3% of *Neospora* positive cases using the anti- *Neospora* (NcSAG1) sera and the anti- *Neospora* (NcSRS2) sera, respectively. Both antisera enable similar identification of *N. caninum* parasites as no significant difference between the results of the two *Neospora* antisera was observed. However, this difference in detection between them could be explained by the fact that the recombinant protein developed for anti-*Neospora* NcSAG1 was only expressed during the tachyzoite stage. The anti-*Neospora* NcSRS2 showed expression during both the tachyzoite and bradyzoite stages, and hence could explain the higher detection achieved in the tissue samples (Gottstein *et al.*, 1998; Hemphill & Gottstein, 2006; Howe *et al.*, 2005; Kasper *et al.*, 1984; Lekutis *et al.*, 2001).

The anti- *Toxoplasma* TgSRS2 and the commercial anti- *T. gondii* IgG only enabled a low detection of *Toxoplasma* parasites, potentially due to the low *T. gondii* prevalence in the samples tested. Both the commercial and the in-house antisera were comparable as no significant difference was detected. Contrary to *Toxoplasma* infections in sheep

and goats, infections in cattle are diagnosed relatively infrequent as clinical signs are rarely exhibited (Dubey, 2010). Similar to our results, a low infection rate of *T. gondii* in cattle was also shown in the study by Hosein *et al.*, (2016) in the UK, which showed that of 305 cattle tested, only 1.6% were positive by PCR for *Toxoplasma*. On the other hand, *Neospora* infection in sheep is more easily diagnosed as the clinical signs are usually more obvious (Buxton *et al.*, 2001; Gonzalez-Warleta *et al.*, 2014; Hartley & Bridge, 1975).

Even though *N. caninum* is mainly associated with cattle and *T. gondii* is mainly associated with sheep, studies have shown findings of *Toxoplasma* in cattle and *Neospora* in sheep (Amdouni *et al.*, 2017; Buxton *et al.*, 1997; Canada *et al.*, 2002; Dubey & Lindsay, 2006; Dubey & Schares, 2011; Hassig *et al.*, 2003; Moreno *et al.*, 2012; Shaapan, 2016). *Neospora* is known to be a specific cause of abortion in 18-20% of cattle in the UK, and it represents a major problem in cattle industries due to its efficient transmission causing significant economic losses and reduction in production efficiency (Guido *et al.*, 2016; Reichel *et al.*, 2013). Similar findings of *Neospora* infections have also been demonstrated in our results. Corresponding to *Neospora* in cattle, *Toxoplasma* plays a major role in abortions of sheep, and it has been estimated that in the UK alone 0.5 million lambs are lost each year due to toxoplasmosis (Innes *et al.*, 2009).

None of the 81 bovine samples were found to be positive for *Toxoplasma* infection by IHC, and only one of the 26 ovine samples tested by IHC were positive for *Neospora* parasites. This potentially indicates that *T. gondii* infections in cattle and *Neospora* infections in sheep are not widespread within Scotland. A study in the UK by Mitchell *et al.* (2017) detected *N. caninum* infections in two lambs during the lambing season in two geographically distant areas of Great Britain by PCR and IHC. Our results and those of Mitchell *et al.* (2017) suggest that *Neospora* in sheep in the UK is only rarely detected, although there are various reports of its importance in sheep flocks as a cause of reproductive failure, as reported in other countries (Gonzalez-Warleta *et al.*, 2014). This was described in the study by Gonzalez-Warleta *et al.* (2014), who detected *N. caninum* DNA in 13 out of 14 still born lambs, and demonstrated that the characteristic lesions caused by *Neospora* were associated with reproductive losses in this sheep

flock. To date, only limited information on *Neospora* infection in sheep has been recorded in the UK, and further studies need to be performed to evaluate the prevalence of *N. caninum* in sheep.

The results in this study demonstrated that 6.5% of ovine and bovine muscle samples showed cysts, yet no labelling was observed with either *Neospora* or *Toxoplasma* antisera, suggesting that they may have been *Sarcocystis* spp.. As it was not possible to produce a genus-specific antiserum against *Sarcocystis*, it was not possible to investigate this possibility by IHC. The PCR assay developed in Chapter 2 enabled detection of *Sarcocystis* spp. DNA. Samples that showed cysts in the tissue samples and no labelling was seen by IHC using *Neospora* and *Toxoplasma* antibodies, were tested with the PCR assay to demonstrate the presence of the *Sarcocystis* spp., in the tissue section. Results confirmed the detection of *Sarcocystis* DNA (*S. gigantea*, *S. cruzi* and *S. tenella*), suggesting that using the PCR analysis along side the IHC assay enables a more accurate identification of protozoan parasites. Similar results were seen when comparing the PCR assay with the newly developed IHC assay, as cases formerly attributed to one protozoan were now associated with another and mixed infections were identified that were previously attributed with a single protozoan parasite. From the comparison of PCR and IHC assay it can be suggested that the PCR analysis may confers a higher specificity in comparison to the Moredun In-house IHC antisera, as it enables a more accurate detection for each protozoan parasite present. However, an accurate comparison test between the PCR and IHC to determine sensitivity is still needed to be carried out using known positive and negative samples. Unfortunately, due to time limitations, this was not carried out, and no such evaluation was done, yet further evaluations are needed.

Furthermore, our results showed a large number of samples were negative for *Neospora* and for *Toxoplasma*, as no labelling was observed. Moreover, 7 cases positive with anti- *Neospora* (NcSAG1) sera were negative with the old *Neospora* antisera and 24 cases positive with the Moredun In-House *Neospora* antisera were negative with the anti- *Neospora* (NcSAG1). Similar results were seen for anti-*Neospora* (NcSRS2) sera, showing 8 cases positive with anti- *Neospora* (NcSRS2) but negative with the Moredun In-House antisera and 22 cases were shown positive with

the Moredun In-House *Neospora* antisera were negative with the anti- *Neospora* (NcSRS2) sera.

In our study, only a single block was used from a selection of multiple blocks from the same animal. Hence, it could be said that this particular section of this block / tissue was negative, but it does not necessarily indicate the absence of the parasite as only one section of one block was tested. It was shown that certain areas of the brain showed a higher density of parasites compared to others. Similar results were observed in the study by Benavides *et al.*, (2011), who showed that tissue cysts and antigen were more frequently recorded in the forebrain and midbrain. Hence, the chances of labelling and detecting *N. caninum* or *T. gondii* using a single tissue block can be low, and the true prevalence of these protozoans in ruminants could be underestimated. From our study, the high percentage of negative results could be explained by only testing one section from one block with each antiserum, potentially resulting in false negative diagnostics results. For diagnostic purposes, consideration should hence be given to the use of multiple sections per block and several blocks. Moreover, blocks where the protozoan parasites are most likely to occur should be tested more frequently to decrease false negative results. However, in this study, this was not done due to the time and cost limitations.

The decreased sensitivity can however be explained, as in this study, only one 4µm section was cut from a single block (and not always from the same tissue block) decreasing the chance of parasite detection considerably as parasite distribution is known to be low and unequal in the tissues used (Dubey & Lindsay, 2006). Moreover, tissue morphology changes considerably with each new section cut, and parasites seen in one section might be absent in the following section, especially as these protozoan parasites can be smaller than 4 µm. Differences in parasite detection can be seen from the comparison of the PCR results and the IHC analysis using the antisera raised against recombinant proteins as not all positive results by IHC were positive by PCR. The study by Nishimura *et al.*, (2013) using cattle experimentally infected with *Neospora*, calculated that 1 g of tissue sample would give roughly 2500 pieces when sliced into 4µm thick sections. When the parasites are evenly distributed, it would take at least 23 sections to find a single parasite (Nishimura *et al.*, 2013). Findings from our

results could be explained as not the same sections were tested by PCR and IHC hence giving variable results of cases being positive by IHC but negative by PCR and vice versa. In general, protozoan parasites are unevenly distributed in tissue samples, making detection of the parasites from fixed tissue sections significantly more difficult. Furthermore, even if lesions are present in the tissue, it does not automatically indicate the presence of the parasites, as lesions are not always associated with the parasites (Benavides *et al.*, 2011). Similar results were seen for the distribution of *T. gondii* in experimentally infected lambs that were infected to assess the presence and distribution of lesions and parasites within different organs (Benavides *et al.*, 2011). In this study, it was observed that the bradyzoites were present in all infected animals, yet the parasites were not always within the lesions themselves (Benavides *et al.*, 2011). It could be possible that the parasites have already been cleared, leading to the differences in the distribution of the lesions and the parasites, which was demonstrated in the study by Nishimura *et al.*, (2013).

Results were compared with the Moredun In-house antisera (produce from whole parasite lysate) and the antisera produced in this study (produced from recombinant proteins) and results showed a higher number of positive sample using the Moredun In-house antisera, suggesting that the newly developed antisera have a lower sensitivity. However, as not all information was available from the previous studies no accurate comparison could be made and further studies are needed using known positive and negative control sample to determine sensitivity of each antisera.Due to the fact that some samples previously shown negative were positive and vice versa suggest that there is some discrepancy between our results, potentially due to decreased sensitivity with cutting different sections for each test performed. On the other hand, from the results it was shown that the Moredun In-house antisera were shown to be un-specific as cross-reactivity was observed whereas the newly developed antisera were shown to be specific, suggesting that these antisera have shown to be an improvement in terms of specificity in the identification of protozoan parasites. As a conclusion the first part of Chapter 4 showed the successful production of genus-specific polyclonal serum raised against *Neospora* and *Toxoplasma* using recombinant proteins. The polyclonal sera were evaluated for functionality and specificity, and showed that they are feasible for the diagnosis of abortion in ruminants as the antisera

enabled a better discrimination of the specific agent involved as cross-reactivity was reduced. However, sensitivity of each antisera are still needed to be confirmed, as no conclusion could be drawn from these resuls of the newly developed antisera and the Moredun In-house antisera and further tested are needed to evaluate sensitivity of the assay.

4.4.2. Monoclonal antibody project

In the second part of Chapter 4, monoclonal antibodies were raised against the recombinant proteins produced in Chapter 3. Even though each of the polyclonal sera raised against recombinant proteins was shown to be specific and can be implemented in the diagnosis of ruminant abortion cases, polyclonal antisera have some limitations. Polyclonal antibodies tend to be less reproducible due to their batch to batch variability (different reactivity) compared to monoclonal antibodies (Kim *et al.*, 2016). There is no guarantee when generating more polyclonal sera against the same recombinant protein that it confers the same specificity, and would need to be re-evaluated to test these properties, which can be very time consuming and costly. Moreover, polyclonal antisera tend to show a higher amount of background due to the natural antibodies in rabbits, which was also shown during our study and can be problematic (Bordeaux *et al.*, 2010; Kim *et al.*, 2016).

A more reliable solution was sought, and monoclonal antibodies raised against the recombinant proteins were produced. Monoclonal (monovalent) antibodies have a greater epitope specificity and show less unspecific binding compared to the polyclonal antisera. Once a specific antibody is generated, it can be produced from hybridoma clones countless times with the same properties, giving it better reproducibility (Greenfield, 2014; Liu, 2014; Zhang, 2012). Due to cost limitations, only the production of two monoclonal antibodies could be attempted. Monoclonal antibodies were raised against *Toxoplasma* rTgSRS2 and against *Neospora* rNcSRS2. The recombinant protein rNcSRS2 was chosen due to its stage-specific expression at both the tachyzoite and bradyzoite stages, compared to rNcSAG1, which is only expressed during the tachyzoite stage (Fuchs *et al.*, 1998; Hemphill & Gottstein, 1996; Hiasa *et al.*, 2012). For the production of monoclonal antibodies, BALB/c mice were

chosen, as they serve as a successful fusion partner with the BALB/c myelomas, resulting in hybridomas that can be grown as tumours producing ascites (Greenfield, 2014; Zhang, 2012).

Four mice per antigen were used in this study, and the immunisation schedule was the same schedule used for the polyclonal rabbit antisera production, as this was previously successful. Similar to the production of rabbit polyclonal antisera, all four *Neospora* mouse polyclonal sera were shown to be specific, as no cross-reactivity was observed with the *Toxoplasma* antigen using ELISA analyses or *Toxoplasma* parasites using IHC analyses. Only the mice showing the highest labelling intensity were taken forward for the production of *Neospora* hybridomas to ensure high labelling intensity of the hybridoma clones.

For the *Toxoplasma* monoclonal production, two mice cross-reacted with *Neospora* antigen during ELISA analyses, and so were not taken forward due to potential cross-reactivity with *Neospora* at later stages. The other two mice were taken forward as no cross-reactivity was observed with *Neospora* in both ELISA and IHC analysis. Multiple epitopes in the polyclonal sera could potentially explain the cross-reactivity of *Toxoplasma* polyclonal sera with *Neospora* antigens. Immunisation with a specific antigen will enhance antibodies against this specific antigen, but could potentially enhance the antibodies that are already present in their system, which could explain why two of the mice polyclonal sera immunised with the *Toxoplasma* recombinant protein showed reactivity against *N. caninum* antigen. The mouse pre-immune sera could not be tested prior to immunisation, and it was not possible to verify what cross-reactivity was already present before immunisation (Abcam; Lathrop *et al.*, 2006).

Following selection of the mice, the spleens producing B- lymphocytes and specific epitopes were fused with myeloma tumour cells developing parental hybridomas (Greenfield, 2014; Zhang, 2012). A successful fusion can result in the production of 200 to 20,000 hybridoma clones (Zhang, 2012), and in our study 2350 hybridomas for each project (*Neospora* and *Toxoplasma*) were developed. The culture supernatants from the fusion plates were initially screened for positive hybridoma clones, as not all hybridomas will be positive when screened, and only positive clones should be

expanded (Zhang, 2012). False positive and nonspecific antibodies need to be eliminated to reduce the number of cultures that need to be maintained (Greenfield, 2014; Zhang, 2012). This was seen in our results as a total of 55 *Neospora* hybridoma clones were positive and reacted with *Neospora* antigen, and 25 *Toxoplasma* hybridoma clones reacted with *Toxoplasma* antigen by ELISA. A total of 5 *Neospora* clones showed cross-reactivity with *Toxoplasma* antigen, and 3 *Toxoplasma* clones showed reactivity with *Neospora* antigen by ELISA. Cross-reactive clones were not taken forward to limit cross-reactivity potential at a later stage. Hybridomas contain a mixture of antibody-producing cells derived from different B-lymphocytes clones, of which each clone can secrete its individual specific antibody, which may explain why some hybridoma clones react or showed cross-reactivity to certain epitopes and others did not (Greenfield, 2014; Liu, 2014).

Even though 37 *Neospora* hybridoma clones and 18 *Toxoplasma* clones remained positive by ELISA, only three *Neospora* and two *Toxoplasma* parental hybridoma cell lines were taken forward for subcloning by limiting dilution. This was done to obtain a homogeneous culture of cell clones secreting one specific monoclonal antibody (Goding, 1996; Zhang, 2012) as they were the only clones positive by IHC. The hybridoma clones showed different reactivity using ELISA and IHC techniques. Possible reasons why some clones are positive by ELISA but negative by IHC could be due to the masked antigens during fixation, protein to protein interactions and the development of secondary structures. This might limit some hybridoma cell supernatant to bind to their target antigen as each cell line expressed antibodies for potentially different epitopes. ELISA uses whole parasite antigen of which each target antigen is visible to the hybridoma clones, which could explain the higher number of positive hybridoma clones using ELISA compared to using the IHC method.

The two *Toxoplasma* parental clones (FD11 and LF11) and one *Neospora* parental clone (AB1) lost reactivity against the *Toxoplasma / Neospora* antigen by ELISA. This could be due to instability and non-clonality of the hybridoma caused by mutation, chromosome losses or due to other variables arising during the process of hybridoma production (Castillo *et al.*, 1994). Studies have shown that hybridoma clones can often lose their specific reactivity due to the appearance of none antibody-producing

hybridoma cells in the culture. Non-producing cells are thought to be due to mutation or the loss of the genes associated with antibody regulation and synthesis, which are usually irreversible (Lee & Palsson, 1993). Hybridoma cultures can become rapidly overgrown by non-secreting / non-producer cells due to their growth advantage over producing hybridomas cells (containing the target epitopes) (Lee & Palsson, 1993). The parental clones were not sub-cloned immediately as they had to be tested for functionality and specificity using IHC analysis. Non-producing cells are not able to take over the whole culture in a short period of time due to limited growth of the entrapped cells, and hence why subcloning should be performed as soon as possible to ensure a stable cell line (Greenfield, 2014; Lee & Palsson, 1993; Zhang, 2012). As a result, no *Toxoplasma* monoclonal antibody could be developed.

The *Neospora* parental hybridoma clones (ME7 and GF7) that remained positive by ELISA were sub-cloned, and for each parental clone, three sub-clones were selected for testing. The *Neospora* GF7 sub-clones however, showed a loss in reactivity by IHC compared to the *Neospora* ME7 sub-clones, and hence were discarded. A loss in reactivity could be explained as certain epitopes are masked due to the fixation of the tissue which could have masked the specific epitope recognized by the GF7 sub-clones. The ME7 sub-clone B12 was further re-cloned, and ME7.1.B12.C9 was chosen, expanded and the supernatant was collected containing the monoclonal antibody. As the sub-clone B12 was previously shown to be specific to *Neospora*, it was expected that the antibody was correspondingly specific. This was demonstrated as the *Neospora* monoclonal antibody ME7.1.B12. C9 only showed specific labelling to *Neospora* when tested against *Neospora*, *Toxoplasma* and *Sarcocystis* control tissues using IHC analyses.

The validation of this monoclonal antibody is needed to confirm its usefulness in IHC diagnostics. This could not be done due to time limitations of this study. The second part of the chapter discussed the successful development of a *Neospora* monoclonal antibody that could be used in the diagnosis of ruminant abortion cases. The IHC technique was optimised to enable specific and sensitive detection of the protozoan parasites *Neospora* and *Toxoplasma* parasites in fixed tissues. However, limitations of the technique, such as only using one section per one block, reduces the diagnostic

sensitivity of IHC analysis (Hardy *et al.*, 2013b; Kim *et al.*, 2016). IHC analysis is a less sensitive diagnostic technique, and should be used in conjunction with other techniques, such as histopathology, PCR or ELISA. A PCR assay, such as the one developed in Chapter 2 of this PhD study, could enable detection and differentiation of protozoan DNA of the genera *Toxoplasma*, *Neospora* and *Sarcocystis*. This PCR assay could be used alongside the IHC technique to verify the presence of protozoan parasites as a cause of abortion.

Chapter 5.

5.1. General Discussion

Neospora caninum, *Toxoplasma gondii* and *Sarcocystis* spp. are closely related intracellular protozoan parasites known to cause neosporosis, toxoplasmosis and sarcocystosis. *Toxoplasma* and *Neospora* are major causes of abortion in livestock worldwide leading to substantial economic losses. *Toxoplasma* is well-known for infections in sheep, but also a wide range of warm-blooded animals including humans. *Neospora* is mainly known as a cause of disease in cattle: however, infections in other warm-blooded animals have been found. *Sarcocystis* is known to infect a wide range of intermediate and definitive hosts including cattle and sheep, of which some *Sarcocystis* species have shown to be more pathogenic than others. Although abortions caused by these protozoan parasites are a major problem for livestock operations and animal welfare worldwide, the identification of a specific cause is particularly difficult and achieved in less than 50% of cases, even in well-established diagnostic laboratories.

Accurate monitoring programs are required to distinguish and determine the cause of abortion, in order to adopt the most relevant disease control strategy. It is necessary to have access to specific diagnostic tests to confirm the presence or rule out the presence of *T. gondii*, *N. caninum*, and *Sarcocystis* spp. as the cause of abortion. However, diagnosis was hindered by the absence of reliable diagnostic methods as discussed in Chapter 1. Detection using existing antibodies against the protozoan parasites *T. gondii*, *N. caninum* and *Sarcocystis* spp. had shown cross-reactivity, which resulted in an inaccurate identification (Chapter 4). The absence of a reliable diagnostic method, therefore, hinders the implementation of accurate treatment measures, monitoring programs and disease control strategies.

A positive diagnostic histopathological test from a cow that has aborted should implicate *N. caninum* as the cause of abortion, and a positive test from a sheep that has aborted would normally indicate *T. gondii* as the cause. However, infections of *T.*

gondii in cattle and *N. caninum* in sheep cannot be ruled out, as these protozoan parasites have been found in a variety of different hosts and can cause clinical disease in other ruminant species (Chapter 1). *Neospora, Toxoplasma*, and *Sarcocystis* share many common morphological and biological similarities, making a differentiation by microscopy between the protozoan parasites challenging. Similarities can not only be seen in the morphology of tissue cysts using histopathology, but also in the genetic and molecular makeup of the protozoan parasites. These similarities were noted throughout Chapters 2, 3 and 4. However, differences observed in the genome were used to develop specific diagnostic tools for the differentiation between *Neospora*, *Toxoplasma* and *Sarcocystis* spp.

There were two main aims of this study; Firstly, to produce genus-specific antibodies raised against recombinant proteins of *T. gondii, N. caninum* and *Sarcocystis* spp. and secondly to produce genus-specific PCR products to be able to accurately differentiate between the protozoan parasites. These aims were achieved throughout this study using a variety of molecular and immunological methods using the genomic information available on these parasites.

The first part of aim 1 (Chapter 3) was achieved and showed the successful development of recombinant proteins for *Neospora*, *Toxoplasma* and *Sarcocystis*. These recombinant proteins can be used to develop genus-specific diagnostic assays, such as IHC, ELISA, or Immunofluorescences that specifically recognised either *Neospora, Toxoplasma* or *Sarcocystis neurona*. Previously studies have shown that the development of recombinant proteins provide an excellent tool for the differentiation and detection of protozoan parasites, such as *Toxoplasma, Leishmania, Trypanosoma* and *Plasmodium* (Farahmand & Nahrevanian, 2016; Hehl *et al.*, 1997; Kimbita *et al.*, 2001; Linh *et al.*, 2017; Luquetti *et al.*, 2003; McAllister, 2016; Reid *et al.*, 2012). For this study, genus-specific recombinant proteins were used instead of whole parasite lysate, to raise genus-specific anti-sera and antibodies in a diagnostic IHC assay for the differentiation of *Neospora, Toxoplasma* and *Sarcocystis* (Chapter 4). The recombinant protein aided in the development of improved and more specific antisera that was able to successful discriminate between *N. caninum* and *T. gondii*.

There are some limitations when testing specificity of the recombinant proteins. One of those limitations is testing the recombinant proteins for specificity using the western blot analysis. Cross-reactive analysis can mainly be performed if positive *Toxoplasma*, *Neospora* and *Sarcocystis* immune sera are available for testing. One of the main challenges seen in the production of recombinant proteins, described in Chapter 3, was the lack of genomic information available for *Sarcocystis* protozoan species. No information on potential surface gene targets for *S. tenella* was available, which meant that primers were designed using target genes from *S. neurona*, where genomic information was more readily available. *Sarcocystis tenella* is the most pathogenic species found in sheep in the UK and was therefore chosen as a target for the development of *Sarcocystis* specific antibodies. However, as only limited information was available no recombinant protein for *S. tenella* was developed. The production of recombinant proteins from specific antigens could be improved if the genetic information was more readily available for certain parasite species. A way to gather more information on pathogenic species could be achieved by the whole-genome sequencing of *S. tenella* or other pathogenic species known to cause disease.

An additional limitation observed in Chapter 3 was the quantity of recombinant protein produced for each target proteins. Even though recombinant proteins were successfully expressed and purified, some proteins had insufficient quantities for immunisation of rabbits, potentially due to the toxic effect of the surface antigen on the bacterial expression system. Surface antigens have been known to be toxic when induced, causing insufficient production of recombinant protein to be expressed in *E. coli* (Akiyama, 2009; Carrio & Villaverde, 2002; Samuelson, 2011; Wagner *et al.*, 2007). If toxic, one might see growth arrest of bacteria .i.e. they stop growing but there could also be problems due to different codon usage between parasites and bacteria. Developing better expression systems in either bacteria or other expression vectors for improved production of highly toxic cells could improve the quantity of expression.

The second part of aim 1 (Chapter 4) showed the successful development of both genus-specific polyclonal antisera and a monoclonal antibody raised against the recombinant proteins developed in Chapter 3. Here three polyclonal sera, two polyclonal anti-*Neospora* sera (NcSRS2 and NcSAG1) and one anti-*Toxoplasma*

serum (TgSRS2), and one *Neospora* specific monoclonal antibody, were generated. The polyclonal antisera enable a genus-specific detection and differentiation of *Neospora* and *Toxoplasma* parasites from both experimentally and naturally infected ruminant samples. The genus-speific antisera enabled an improvement to the IHC assay by better and more specific discrimination of protozoan parasites due to reduced cross-reactivity. However more tests are needed to evaluate sensitivity of each antisera produced. The polyclonal sera and monoclonal antibody could used in IHC analysis to achieve a more specific diagnosis of protozoan infections. Previous diagnoses at Moredun used antisera (commercial or in-house) raised against whole parasite lysates and had shown to result in cross-reactivity with *Neospora*, *Toxoplasma* and *Sarcocystis.* Using whole parasite lysates to raise antisera with closely related parasites facilitates the detection of multiple antigens and hence explains the non-specificity of these antisera. However, when using recombinant proteins, one can limit the detection to a single antigen (recombinant protein). This supports the results of many studies that have demonstrated that using whole parasite lysates showed cross-reactivity with closely related parasite species, whereas using recombinant proteins cross-reactivity was eliminated (McAllister *et al.*, 1996b; Peters *et al.*, 2000; Van Maanen *et al.*, 2004).

The development of rabbit polyclonal sera was initiated by screening the rabbit pre-immune sera to choose the best candidate rabbits for immunisation with recombinant proteins. This has shown to be an important process for the generation of specific antibodies, as the pre-immune rabbit sera reactivated with protozoan parasites. This study has shown that non-specific antibodies, targeting protozoan parasites, were already present in the majority of the available rabbits and that rabbits need to be carefully selected prior to immunisation. Moreover, the non-specific reactivity of polyclonal mouse sera was also observed during the monoclonal antibody development. The mice (Balb/c) used, come from pathogen-free breeding colonies, making exposure to protozoan parasites unlikely, yet reactivity of two mice was observed with *Toxoplasma* and *Neospora*. It is likely that the animals have antibodies against other biological material that either recognise the parasites epitopes directly or non-specifically cross-react with parasites epitopes. The same can be said for the rabbit polyclonal pre-immune sera. In order to develop specific antisera / antibodies, cross-reactive epitopes should be avoided, and the pre-screening of pre-immune sera should

be done to limit the potential of cross-reactive antibodies. Non-specific antibodies already present in the rabbit or mice pre-immune sera could be enhanced post immunisation increasing the chances of cross-reactivity.

Nevertheless, using polyclonal rabbit sera has limitations. Using polyclonal rabbit antisera for the development of diagnostic antibodies is less reproducible, as it has a batch to batch variability. When a new batch of polyclonal sera is produced, it has to be tested for specificity, as there is no guarantee that it confers the same specificity (Kim *et al.*, 2016). Rabbit polyclonal sera shows high levels of background staining which may make the diagnosis more challenging, as observed in this study. In the second part of Chapter 4, monoclonal antibodies were produced using the recombinant proteins produced in Chapter 3. Monoclonal antibodies are known to be more specific and have better reproducibility, as the hybridoma clones generating the specific antibody, can be regrown countless times with the same characteristics as the previous batch (Greenfield, 2014; Liu, 2014; Zhang, 2012) and therefore a monoclonal antibody (ME7.1.B12.C9) was developed.

Results in Chapter 2 showed the successful development of a variety of PCR arrays that enable species-specific and genus-specific detection and differentiation of DNA from *Toxoplasma*, *Neospora* and *Sarcocystis* spp. using nested PCRs. These PCR assays could be used for the diagnosis of protozoan parasites (*Neospora*, *Toxoplasma* and *Sarcocystis* spp.), from fixed and fresh tissue of a variety of host samples. Previous studies have shown that fixing tissues leads to fragmentation of the nucleic acids making amplification of larger DNA fragments difficult (Gilbert *et al.*, 2007; Kokkat *et al.*, 2013; Ren *et al.*, 2000). However, the PCR primers developed enabled the amplification using a smaller DNA fragment making amplification of DNA from fixed tissues possible, which has also been shown in previous studies (Lin *et al.*, 2012). The regions used (ITS1 region and rRNA gene) have shown to be suitable candidates for diagnosis and enabled successful amplification of apicomplexan DNA from *Toxoplasma*, *Neospora* and *Sarcocystis* spp. utilising polymorphic and conserved regions in these targets (Dubey & Schares, 2006; Ellis, 1998; Fischer & Odening, 1998; More *et al.*, 2008; Neefs *et al.*, 1991). The PCR assay was shown to be an improvement for the identification of protozoan parasites using fixed tissue samples.

Primarly, due to it's enhanced identification of protozoan parasites and better discrimination of specific protozoan parasites that were formerly attributed to one protozoan and are now associated with another. And secondly due to an improvement of idenficiation of mixed infections that were previously attributed to a single parasite. However, more tests are needed to further evaluate sensitivity.

There were some limitations in the development of genus-specific PCR primers. One of the main limitations was the sequence information available at the time for different sequences of the protozoan species studied in this thesis. The majority of information available was for the 18S rRNA gene, especially for *Sarcocystis* spp. Only limited information for *Sarcocystis* spp. regions other than the 18S rRNA gene, such as ITS1 regions, was available at the time of primer design. Target regions and primer design were limited to what genetic information was available for each species at the time of development. Even though the 18S rRNA gene has shown polymorphism among the protozoan species, differentiation between very closely related *Sarcocystis* species, and between *Neospora* and *Toxoplasma* were problematic. The ITS1 regions have shown more polymorphism, and primer design using this region was able to differentiate between *Neospora* and *Toxoplasma*. However, no genus-specific PCR primers for the genus *Sarcocystis* were developed due to their highly polymorphic regions, and it was not possible to design primers to amplify a small enough DNA fragment to enable amplification from fixed tissue samples. Chapter 2 of this thesis demonstrated the knowledge gap of sequence information available in databases. At the start of primer design, only limited information on other target regions, such as ITS1 and COX was available for primer design (Gjerde *et al.*, 2015). With continous development and advances in information available in the databases, an improved PCR diagnostic assay for the diagnosis and differentiation of very closely related species could be achievable in the near future.

One of the main challenges for the development of genus-specific PCR primers was testing the specificity of each primer. Having the correct positive control material for the amplification by PCR was problematic, as DNA samples positive for certain parasite species had to be acquired first. Only once the positive control material was available the PCR primers could be tested for specificity, in particular for *Sarcocystis*

spp. However, not all *Sarcocystis* spp. used in the primer design were tested for specificity, as only a few *Sarcocystis* DNA samples were available. Chapter 2 is partially based on *in silico* analysis using multiple-sequence alignments and phylogenetic analysis of protozoan species, to predict what might be amplified using the PCR primers. Specificity testing for some protozoan species still needs to be validated once the positive control material becomes available. A potential 'biobank' of resources for researchers of related fields should be made available for protozoan control DNA material and would simplify testing of specificity rather than solely basing it on *in silico* analysis.

Furthermore, in this validation of the PCR methodology, not all cases tested were positive for protozoal DNA, even in cases that were selected based on findings consistent with protozoal infections. A reason for this could be that only a 1g of the fresh or frozen tissue sample was used in the extraction of DNA. The suspected samples where the parasites' DNA is present could have been missed as these protozoan parasites are low and unequally distributed in the tissue samples (Dubey & Lindsay, 2006). Another possible cause of low detection of protozoal DNA from suspected cases, who had been fixed, could be because only one block per suspected case and only 2 x 10µm from the fixed tissue sample was used in the PCR. Even if more than one section is cut, detection of DNA from fixed tissue sections is difficult as the sensitivity considerably decreases due to the unequal distribution of parasite present in the tissues. Ideally, each tissue sample block and a higher amount of tissue sections per case should have been tested to increase both the chance of amplification of DNA from *Neospora*, *Toxoplasma* and *Sarcocystis* spp. and the chance of eliminating false negatives due to the technical limitations of the method.

Lastly, contamination can be a major problem in diagnostic testing, leading to the generation of false positive results. Contamination may confound an accurate diagnosis, even when genus-specific PCR primers are used. The nested PCR assay should be performed with utmost care to avoid contamination. Handling of samples, control material and preparation of reagents should be done cautiously and ideally in separate locations in order to prevent contamination and limit false positive results to achieve a reliable diagnostic test. Diagnostic tests are in constant need of improvement

to enhance the sensitivity and specificity, as false negative and / or false positive results may still occur due to the aforementioned limitations. Identifying contamination issues and thus preventing a false diagnosis could be limited by using different extractions controls, such as using positive and negative control samples for the DNA extractions and PCR amplification.

Overall, this PhD thesis describes the development of diagnostic tools against various parasites using molecular tools and IHC techniques. The study shows that the application of molecular tools can be used for diagnosis of protozoan parasites, which can subsequently lead to optimized treatments, prevention and control methods. Chapter 2 demonstrated the development of PCR primers using various target regions to enable accurate detection of protozoan DNA. Sequence analyses using multi-sequence alignment and phylogenetic analyses demonstrated how closely related these parasites are at a genetic level. Such PCR-based assays enable the simultaneous detection and identification of various parasitic organisms up to species level, not only in clinical samples but also in natural vectors. The continuous development and commercialization of various diagnostic tests can significantly contribute to better disease control and prevention. The PCR assay developed in Chapter 2, could be used for a more precise diagnosis of protozoan parasites. An example of this can be shown in our publication of Lepore *et al.* (2017), where the diagnosis using the PCR primers enabled us to make a precise diagnosis of *Sarcocystis lutrae* in samples that were also positive for *T. gondii* and *N. caninum* DNA, which shows that this species has a more extensive host range other than otters. Diagnosis of protozoan could enable us to gain a better understanding of the distribution of these parasites in hosts not yet identified and help to better understand the distribution and transmission range in a different host species.

Unfortunately, due to time and cost constraints, there were a few studies that could not be carried out and competed. A follow up from this PhD could be the testing, validation, evaluating and comparing of both diagnostic assays, using genus-specific PCR assay and genus-specific antisera / antibodies against the Moredun archived samples to achieve a definite diagnosis. Using known positive and negative controls, one can more accurately test the sensitivity and specificity of the assays. Additionally,

another area to follow up on would be the production of a genus-specific antibody against *S. tenella*, which due to the lack of genetic information available for this species was not achieved. Sequencing more of the genome (i.e. surface antigens) of *S. tenella* or other pathogenic *Sarcocystis* spp. could be used to develop genus-specific antibody. Another study that was not completed due to cost restraints was the development of a monoclonal antibody for *Toxoplasma* as the identification of positive hybridoma sub-clones failed in this study. Ideally, a new group of mice should be immunised with the recombinant protein for the development of more hybridoma clones to verify a positive clone for functionality and specificity in the IHC assay.

The work carried out during this PhD has enhanced our understanding of the production, standardisation and validation of various diagnostic techniques such as IHC and PCR for the genus-specific diagnosis of protozoan parasites and enabled the development of newer and better diagnostic tools. Chapter 3 and 4 provided in-depth detail on the production of genus-specific recombinant proteins using surface antigen targets, for the production of genus-specific antibodies. The results of this study enabled a better understanding of the development of antibodies, and what limitations are to be expected. Moreover, this work has addressed the problem of cross-reactivity of antisera using whole parasite lysates and has overcome this problem by using recombinant proteins. The majority of diagnostic results for ruminant abortions come back as 'undiagnosed', however, the development of these genus-specific antibodies and diagnostic methods can improve specificity of diagnostic cases where protozoan parasites are suspected to be the cause of abortion. Achieving a more specific diagnosis of *Neospora*, *Toxoplasma* or *Sarcocystis* in a herd or flock will enable the farmer or vets to implement specific control measures, such as control of the intermediate hosts, vaccination in case of *Toxoplasma* infections or culling of *Neospora* infected animals to prevent further transmission.

Conventional microscopy does not allow differentiation between protozoan parasites. Using genus-specific antibodies enables a simpler diagnosis of protozoan infections. The tools developed in this study are not limited to diagnosis. They could also be used for research purposes and therapeutics, they could help improve the use of IHC in epidemiological and clinical investigations, and help for example to test the validity of

drugs or treatment methods of the disease. Future work using antibodies developed in this study could be used for epidemiological studies of parasite distribution within herds and flocks, as well as determining the significance of mixed protozoal infections in ruminant species.

The work in this study has demonstrated how various target genes and regions can be exploited for the development of diagnostic tools. Further studies may focus on the development of mAbs and pAbs from recombinant proteins targeted to other coccidia, such as *Besnoitia* spp., *Hammondia* spp., or *Sarcocystis* species, once suitable gene targets become available. The reagents developed during the course of this study are not limited to PCR and IHC, and in the future could be used in other methods such as ELISA's, immunofluorescence or fluorescence-activated cell sorting (FACS). In addition, the antisera could be used to authenticate successful *Neospora* infections in Vero cell lines from lyophilized material using immunofluorescence labelling methods. However, such studies are not limited to *Neospora, Toxoplasma* or *Sarcocystis* and specific antibody development against recombinant proteins could be applied to other vaccine candidates from other parasites known to cause clinical disease.

5.2. PhD Outcomes:

This study describes the development of specific diagnostic tools for the detection of protozoan infections of ruminants.

The main outcomes from this thesis are:

- Successful identification of target regions used for primer design for genus-specific PCR. (Chapter 2).
- Successful development of genus-specific PCR assays for the detection and differentiation of DNA from the protozoan *T. gondii, N. caninum* and a variety of *Sarcocystis* spp. (Chapter 2).
- Improved diagnosis by PCR of protozoan parasites (*Neospora, Toxoplasma* and *Sarcocystis* spp.) from fixed and fresh tissue material. (Chapter 2).
- Successful identification of target regions for the production of genus-specific recombinant proteins for *Neospora, Toxoplasma* and *Sarcocystis* spp. (Chapter 3).
- Successful expression of surface antigen and surface related sequence from *Neospora, Toxoplasma* and *Sarcocystis neurona* (Chapter 3).
- Successful expression and purification of genus-specific recombinant proteins for *Neospora, Toxoplasma* and *Sarcocystis* spp. for the development of genus-specific antibodies (Chapter 3).
- Successful development of genus-specific polyclonal rabbit antisera for the genus-specific detection of *Neospora* and *Toxoplasma*. (Chapter 3 and 4).
- Improved detection of the protozoan parasites *Neospora* and *Toxoplasma* using *Neospora* and *Toxoplasma* genus-specific antisera. (Chapter 4).
- Successful development of a *Neospora* genus-specific monoclonal antibody that can be used for the diagnosis of *N. caninum*. (Chapter 4).

Chapter 6. References

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Chapter 7. Appendices

Appendix I. Tables

APPENDIX TABLE I. PROTOZOAL DNA TARGETS USED FOR MULTI-SEQUENCE ALIGNMENTS AND PRIMER DESIGN.

		Species		
18S rRNA gene	*Sarcocystidae*	*N. caninum*	*T. gondii*	
		S. lacerate	*S. alces*	*S. mucosa*
		S. rangiferi	*S. grueneri*	*S. hircanis*
		S. silva	*S. arieticanis*	*S. bovini*
		S. tarandi	*S. hjorti*	*S. hominis*
		S. sinensis	*S. cruzi*	*S. levinei*
		S. hirsuta	*S. rangi*	*S. heydorni*
		S. buffalonis	*S. alceslatrans*	*S. fusiformis*
		S. moulei	*S. capreolicanis*	*S. fayeri*
		S. scandinavica	*S. hardangeri*	*S. gigantea*
		S. tarandivulpes	*S. oviformis*	*S. rileyi*
		S. tenella	*S. ovalis*	*S. lutrae*
		S. capracanis	*S. neurona*	*S. gracilis*
	Closely related	*H. hammondia*	*H. heydorni*	*H. triffittae*
		Cryptosporidium parvum	*Theileria annulata*	*Besnoitia besnoiti*
	Unrelated	*Liriodeodron tulipifera*	*Yeast*	
	Host	*Bos taurus*		
ITS1 region	*Sarcocystidae*	*N. caninum*	*T. gondii*	
		S. cruzi		
		S. fusiformis	*S. buffalonis*	
		S. hirsuta	*S. bovini*	
		S. tenella	*S. sinensis*	
	Closely related	*H. hammondia*	*H. heydorni*	*H. triffittae*
		Cryptosporidium parvum	*Theileria annulata*	*Besnoitia besnoiti*
	Host	*Bos taurus*		

APPENDIX TABLE II. LIST OF PROTOZOAN SPECIES ACQUIRED DURING THIS STUDY

Source	Region	Species DNA	From	Stock generated	Accession Number
Moredun Research Institute	UK	*N. caninum*	(NC1) cell culture tachyzoites	18S/ITS1	L24380
Moredun Research Institute	UK	*T. gondii*	(M4)/(S48) cell culture tachyzoites	18S/ITS1	L24381
Moredun Research Institute	UK	*S. tenella*	Diagnostic sheep	18S	L24383
Moredun Research Institute	UK	*S. fayeri*	Diagnostics horses	18S	AB661447
Moredun Research Institute	UK	*S. tenella*	Ovine dissection MP15/847 and MP15/848	18S	NA
Moredun Research Institute	UK	*S. lutrae*	Diagnostics badgers	18S/ITS1	KM657769
Moredun Research Institute	UK	*S. rileyi*	Diagnostics Capercaillie	18S	KJ396583
Moredun Research Institute	UK	*S. gigantea*	Ovine SRUC A-3, B-1, B-5, B-6	18S	L24384
Jitender. P. Dubey	USA	*S. neurona*	(SN37R) cell cultured merozoites	18S	U07812
Bjorn K. Gjerde	Argentina	*S. bovifelis*	Cattle B4.24	18S	KT901135
Bjorn K. Gjerde	Argentina	*S. cruzi*	Cattle B4.13	18S	JX679468
Bjorn K. Gjerde	New Zealand	*S. hirsuta*	Cattle B6.4	18S	NA
Bjorn K. Gjerde	Germany	*S. hirsuta*	Cattle B12.2	18S	JX855283
Bjorn K. Gjerde	New Zealand	*S. bovini*	Cattle B8.4	18S	KT901155
Bjorn K. Gjerde/ Dr Hilali	Egypt	*S. buffalonis*	Buffaloes Bb18.1	18S	AF01712
Bjorn K. Gjerde/ Dr Hilali	Egypt	*S. levinei*	Buffaloes Bb20.1	18S	KU247922
Bjorn K. Gjerde/ Dr Hilali	Egypt	*S. sinensis*	Buffaloes Bb21.26	18S	JX679466
Bjorn K. Gjerde/ Dr Hilali	Egypt	*S. fusiformis*	Buffaloes Bb21.6	18S	NA
Bjorn K. Gjerde/ Dr Hilali	Egypt	*S. fusiformis*	Buffaloes Bb22.3	18S	U03071
Bjorn K. Gjerde	N/A	*S. fusiformis/S. sinensis*	Experimental infected cats from buffaloes Cat2.6	18S	NA
Bjorn K. Gjerde	N/A	*H. triffiattae*	Experimental infected foxes from moose 2007	18S/ITS1	GQ984222
Gereon Schares	Germany	*H. hammondia*	D151120-10-5 oocysts	18S/ITS1	AH008381
Gereon Schares	Germany	*H. heydorni*	D160072 -10-5 oocysts	18S/ITS1	KT184370
Gaston More	Argentina	*S. rommeli*	1 cyst	18S	NA
Gaston More	Argentina	*S. hominis*	1 cyst	18S	JX679470
Gaston More	South American	*S. aucheniae*	1 cyst -Camelids	18S	AF017123

APPENDIX TABLE III. REPRESENTATIVE OF EACH PROTOZOAN PCR PRIMER COMBINATION FROM THE 18S AND ITS1 REGION, INCLUDING SPECIES AMPLIFIED BY EACH PRIMER PAIR, BASED ON *IN SILICO* ANALYSIS.

Forward	Species specificity	Reverse	Species specificity	Combination	Combination specificity	Base pairs
NTS-S-F1	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsuta, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, S. heydorni, S. bovini, S. hominis, S. hircicanis, S. fayeri, S. levinei, H. hammondia, H. heydorni, H. triffittae, Theileria anulata, C. parvum, Yeast,*	NTS-18S-R1	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsuta, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, S. heydorni, S. bovini, S. hominis, S. hircicanis, S. fayeri, S. levinei, H. hammondia, H. heydorni, H. triffittae*	NTS-18S-F1 + NTS-18S-R1	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsuta, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, S. heydorni, S. bovini, S. hominis, S. hircicanis, S. fayeri, S. levinei, H. hammondia, H. heydorni, H. triffittae*	470bp

NTS-18S-F2	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsuta, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, S. heydorni, S. bovini, S. hominis, S. hircicanis, S. fayeri, S. levinei, H. hammondia, H. heydorni, H. triffittae*	NTS-18S-R2	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsuta, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, S. heydorni, S. bovini, S. hominis, S. hircicanis, S. fayeri, S. levinei, H. hammondia, H. heydorni, H. triffittae, C. parvum*	NTS-18S-F2 + NTS-18S-R2	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsuta, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, S. heydorni, S. bovini, S. hominis, S. hircicanis, S. fayeri, S. levinei, H. hammondia, H. heydorni, H. triffittae*	228bp
Forward	**Species specificity**	**Reverse**	**Species specificity**	**Combination**	**Combination specificity**	**Base pairs**
***Sarcocystis* group primer - Forward specificity**						
S-18S-G1	*S. gigantea, S. moulei*	NTS-18S-R2	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsute, S. buffalonis, S.*	S-18S-G1 + NTS-	*S. gigantea, S. moulei*	~196

			scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, C. parvum	18S-R2		
S-18S-G2	*S. hirsute, S. buffalonis*	NTS-18S-R2	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsute, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, C. parvum*	S-18S-G2 + NTS-18S-R2	*S. hirsute, S. buffalonis*	~230
S-18S-G3	*S. rangiferi, S. tarandi, S. sinensis, S. silvia, S. bovini, S. hominis, S. rommeli*	NTS-18S-R2	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsute, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella, C. parvum*	S-18S-G3 + NTS-18S-R2	*S. rangiferi, S. tarandi, S. sinensis, S. silvia, S. bovini, S. hominis, S. rommeli*	~170
***Sarcocystis* group primer - Reverse specificity**						
S-18S-G6- F	*S. tenella, S. sinensis, S. capracanis, S. tarandivulpes, S. arieticanis, S. grueneri, S. alces, S. capreolicanis, S. rangi, S. cruzi, S. hjorti, S. bovini, S. hircanis, S. levinei, S. alceslatrans. S. fayeri*	S-18S-G6-R	*S. tenella,*	S-18S-G6-F + S-18S-G6-R	*S. tenella,*	~220

NTS-18S-F2	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsute, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella,*	S-18S-G5	*S. tenella, S. arieticanis, S. grueneri, S. alces, S. capreolicanis, S. gracilis, S. alceslatrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. levinei, S. heydorni and S. hircanis,*	NTS-18S-F2 + S-18S-G5	*S. tenella, S. arieticanis, S. grueneri, S. alces, S. capreolicanis, S. gracilis, S. alceslatrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. levinei, S. heydorni, and S. hircanis*	~230
NTS-18S-F2	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsute, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella,*	S-18S-G4	*S. silva, S. tarandi, S. sinensis, S. rangiferi, S. fusiformis, S. moulei, S. gigantea, S. scandinavica, S. oviformis, S. ovalis, S. fayeri, S. hardangeri, S. baffalonis, S. hirsuta, S. hominis and S. bovini*	NTS-18S-F2 + S-18S-G4	*S. silva, S. tarandi, S. sinensis, S. rangiferi, S. fusiformis, S. moulei, S. gigantea, S. scandinavica, S. oviformis, S. ovalis, S. fayeri, S. hardangeri, S. baffalonis, S. hirsuta, S. hominis and S. bovini*	~200
NTS-18S-F2	*T. gondii, N. caninum, B. besnoiti, S. neurona, S. lutrae, S. laverate, S. rileyi, S. mucosa, S. oviformis, S. hardangeri, S. ovalis, S. moulei, S. gigantea, S. fusiformis, S. rangiferi, S. tardandi, S. silva, S. sinensis, S. hirsute, S. buffalonis, S. scandinavica, S. arieticanis, S. gruneri, S. alces, S. alces, S. capreolicanis, S. gracilis, S. alcestrans, S. capracanis, S. rangi, S. cruzi, S. hjorti, S. tarandivulpes, S. tenella,*	S-18S-G9	*S. neurona, S. lutrae, S. lacerate*	NTS-18S-F2 + S-18S-G9	*S. neurona, S. lutrae, S. lacerate*	~160

APPENDIX TABLE IV A. RESULTS FOR THE VALIDATION OF PCR PRIMER ON FIXED TISSUE SAMPLES.

Animal identifier	Host species	Tissue samples	PCR					Sequencing results	Information availabe from the surveillance database		
			18S Pan	18S Neo/ Toxo	ITS1 Neo	ITS1 Toxo	18S Sarco		Tissue	Ab tested	Results
Moredun archived surveillence abortion cases											
MP08/386	Bovine	Brain	-	-	-	-	-	NA	Placenta	anti-Toxo	-
MP08/557	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP08/961	Bovine	Brain	+	+	+	-	-	*N. caninum*	Brain	anti-Neo	-
MP08/941	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP08/1015	Bovine	Brain	+	+	+	-	-	*N. caninum*	Mixed	anti-Neo	Inconc.
MP08/1022	Bovine	Brain	+	+	+	-	-	*N. caninum*	Brain	anti-Neo	-
MP08/1103	Bovine	Brain	+	+	+	-	-	*N. caninum*	Mixed	anti-Neo	-
MP08/1172	Bovine	Brain	+	+	+	-	-	*N. caninum*	Brain	anti-Neo	Inconc.
MP08/1212A	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP08/1211	Bovine	Brain	+	+	+	-	-	*N. caninum*	Brain	anti-Neo	-
MP09/121	Bovine	Brain	+	+	+	-	+	*N. caninum/S. cruzi*	Mixed	anti-Neo	+ *N. caninum*
MP09/122	Bovine	Placenta	+	+	+	-	-	*N. caninum*	Placenta	anti-Neo	-
MP09/274	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP09/819	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP09/831	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP09/1153	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-

MP09/1264B	Bovine	Brain	-	-	-	-	-	NA	Mixed	anti-Neo	+ *N. caninum*
MP09/1264C	Bovine	Brain	-	-	-	-	-	NA	Mixed	anti-Neo	-
MP09/1249A	Bovine	Brain	-	-	-	-	-	NA	Mixed	anti-Neo	-
MP10/471	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP10/838	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP10/890	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP10/1059	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP11/32	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP11/276	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP11/547	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP11/546	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP11/613	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP11/653	Bovine	Heart	-	-	-	-	-	NA	Heart	anti-Neo	-
MP11/0543	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP11/807	Bovine	Brain	+	+	+	-	-	*N. caninum*	Brain	anti-Neo	-
MP11/1077	Bovine	Brain	-	-	-	-	-	NA	Mixed	anti-Neo	-
MP11/1062	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP11/1061	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP12/593	Bovine	Placenta	-	-	-	-	-	NA	Placenta	anti-Neo	+ *N. caninum*
MP12/486B	Bovine	Brain	+	+	+	-	-	*N. caninum*	Brain	anti-Neo	+ *N. caninum*
MP12/0785	Bovine	Brain	-	-	-	-	-	NA	Mixed	anti-Neo	Inconc.
MP12/1316	Bovine	Brain	-	-	-	-	-	NA	Mixed	anti-Neo	-

MP12/1364	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP13/110	Bovine	Brain	-	-	-	-	-	NA	Mixed	anti-Neo	+ *N. caninum*
MP13/677	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Toxo	-
MP13/808	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	Inconc.
MP13/0825	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP14/0685	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	NL
MP14/0706	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP14/0707	Bovine	Brain	+	+	-	+	-	*T. gondii*	Brain	anti-Neo	+ *N. caninum*
MP14/0736	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP14/0797	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP14/0958	Bovine	Brain	+	+	-	+	-	*T. gondii*	Brain	anti-Neo	-
MP15/0006	Bovine	Brain	+	+	+	-	-	*N. caninum*	Mixed	anti-Neo	Inconc.
MP15/0012	Bovine	Heart	+	+	+	-	-	*N. caninum*	Brain	anti-Neo	NL
MP15/0014	Bovine	Heart	-	-	-	-	-	NA	Heart	anti-Neo	Inconc.
MP15/0194	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	NL
MP15/0901	Bovine	Brain	+	+	-	-	-	*N. caninum*	Brain	anti-Neo	-
MP15/0948	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*
MP17/0012	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP17/0013	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP17/0021A	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	-
MP17/0023	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	+ *N. caninum*

MP17/732	Bovine	Brain	-	-	-	-	-	NA	Brain	anti-Neo	NL
MP17/689	Bovine	Brain	+	+	-	+	-	*T. gondii*	Brain	anti-Neo	NL
MP08/286A	Ovine	Placenta	-	-	-	-	-	NA	Placenta	anti-Toxo	-
MP09/118A	Ovine	Brain	+	+	-	+	-	*T. gondii*	Brain	anti-Toxo	-
MP09/118C	Ovine	Brain	+	+	-	+	-	*T. gondii*	Brain	anti-Toxo	-
MP09/131	Ovine	Placenta	-	-	-	-	-	NA	Placenta	anti-Toxo	Inconc.
MP12/287	Ovine	Brain	+	+	-	+	-	*T. gondii*	Mixed	anti-Toxo	+ *T. gondii*
MP13/267A	Ovine	Brain	-	-	-	-	-	NA	Brain	anti-Toxo	-
MP13/267B	Ovine	Brain	+	+	-	+	-	*T. gondii*	Brain	anti-Toxo	-
MP13/267C	Ovine	Placenta	-	-	-	-	-	NA	Placenta	anti-Toxo	-
MP14/0162	Ovine	Brain	-	-	-	-	-	NA	Brain	anti-Toxo	-
MP14/0231	Ovine	Brain	-	-	-	-	-	NA	Brain	anti-Toxo	NL
MP14/0745	Ovine	Brain	-	-	-	-	-	NA	Brain	anti-Sarco	-
MP15/0202	Ovine	Heart	-	-	-	-	-	NA	Heart	anti-Toxo	NL
MP15/0203	Ovine	Heart	-	-	-	-	-	NA	Heart	anti-Toxo	NL
MP15/0966	Ovine	Heart	-	-	-	-	-	NA	Heart	anti-Toxo	NL
MP16/0370B	Ovine	Brain	+	+	-	+	-	*T. gondii*	Brain	anti-Toxo	+ *T. gondii*
MP17/0062	Ovine	Brain	-	-	-	-	-	NA	Brain	anti-Toxo	-
MP17/0094	Ovine	Brain	+	-	-	-	+	*S. tenella*	NA	NA	NA
MP17/0261	Ovine	Brain	+	+	-	+	-	*T. gondii*	Brain	anti-Toxo	NL
MP17/0562	Ovine	Brain	+	-	-	-	+	*S. tenella*	Brain	anti-Toxo	NL
MP17/0630	Ovine	Brain	-	-	-	-	-	NA	Brain	anti-Toxo	NL
MP16/0211	Ovine	Heart	+	-	-	-	+	*S. tenella*	Brain	anti-Toxo	+ *S. tenella*
Samples send to Moredun Pathology											
15231**	Bovine	Heart	+	-	-	-	+	*S. cruzi*	NA	NA	NA

14494**	Bovine	Heart	-	-	-	-	-	NA	NA	NA	NA
16119**	Bovine	Heart	+	-	-	-	+	*S. cruzi*	NA	NA	NA
1828**	Bovine	Heart	-	-	-	-	-	NA	NA	NA	NA
V12A- 09 E01*	Bovine	Heart	+	-	-	-	+	*S. cruzi*	NA	NA	NA
V13A- 09 E01*	Bovine	Heart	+	-	-	-	+	*S. cruzi*	NA	NA	NA
16N09***	Ovine	Heart	+	-	-	-	+	*S. tenella*	NA	NA	NA
MP09/0286*	Ovine	Placenta	+	+	-	+	+	*T. gondii/S. tenella*	Placenta	anti-Toxo	+ *T. gondii*
MP06/0228*	Ovine	Heart	+	-	-	-	+	*S. tenella*	Heart	anti-Sarco	+ *S. tenella*
MP15/0865-A*	Ovine	Heart	+	-	-	-	+	*S. gigantea*	NA	NA	NA
MP15/0866-B*	Ovine	Heart	+	-	-	-	+	*S. gigantea*	NA	NA	NA
MP15/0867-B*	Ovine	Heart	+	-	-	-	+	*S. gigantea*	NA	NA	NA
MP15/0868-B*	Ovine	Heart	+	-	-	-	+	*S. gigantea*	NA	NA	NA
MP15/0847*	Ovine	Heart	+	-	-	-	+	*S. tenella*	NA	NA	NA
MP15/0848*	Ovine	Heart	+	-	-	-	+	*S. tenella*	NA	NA	NA

Legend. NA= not applicable, NL= not listed, += positive results, -= negative results. 18S PAN represents primers (NTS-18S-F1 + NTS-18S-R1 and NTS-18S-F2 + NTS-18S-R2), 18S Neo Toxo represents (NTS-18S-F1 + NTS-18S-R1 and NTH-18S-F2 + NTH-18S-R2), ITS1 Neo represents (NTH-ITS1-F+NTH-ITS1-R and NEO-NP1 + NEO-NP2, ITS1 Toxorepresents (NTH-ITS1-F+NTH-ITS1-R and TOXO-NP1 + TOXO-NP2), 18S Sarco represents (NTS-18S-F1 + NTS-18S-R1 AND S-18S-G1, S-18S-G2, S-18S-G3, S-18S-G4, S-18S-G5, S-18S-G6 and S-18S-G9). MP numbers represents cases uspected of protozoan lesions of protozoan infections from Moredun archive, * represent field cases suspected of protozoan lesions of protozoan infections,** represent cases sent from Uruguay suspected of protozoan lesions of protozoan infections *** represents a case suspected of protozoan lesions of protozoan infections sent from Argentina

APPENDIX TABLE IV B. RESULTS FOR THE VALIDATION OF PCR PRIMERS FROM FROZEN TISSUE SAMPLES.

Animal number	Host species	Tissue samples	PCR					Sequencing results
			18S Pan	18S Neo/ Toxo	ITS1 Neo	ITS1 Toxo	18S Sarco	
S425177.1	Ovine	Brain	+	+	-	+	-	*T. gondii*
		Heart	+	+	-	+	-	*T. gondii*
		Placenta	+	+	-	+	-	*T. gondii*
S425055	Ovine	Brain	+	+	-	+	-	*T. gondii*
		Heart	+	+	-	+	-	*T. gondii*
		Placenta	+	+	-	+	-	*T. gondii*
S425045	Ovine	Brain	-	-	-	-	-	NA
		Heart	-	-	-	-	-	NA
		Placenta	-	-	-	-	-	NA
S424955	Ovine	Brain	-	-	-	-	-	NA
		Heart	-	-	-	-	-	NA
		Placenta	-	-	-	-	-	NA
MP15/0924 A	Bovine	Brain	+	+	+	-	-	*N. caninum*
		Spinal cord	+	+	+	-	-	*N. caninum*
MP15/0924 B	Bovine	Brain	+	+	+	-	-	*N. caninum*
		Heart	+	+	+	-	-	*N. caninum*
		Spinal cord	+	+	+	-	-	*N. caninum*
MP15/0924 C	Bovine	Brain	+	+	+	-	-	*N. caninum*
		Heart	-	-	-	-	-	NA
		Spinal cord	+	+	+	-	-	*N. caninum*
Cappercailies 1	Bird	Muscle	+	-	-	-	+	*S. rileyi*
Cappercailies 2	Bird	Muscle	+	-	-	-	+	*S. rileyi*
BL230	Bat	Muscle	-	-	-	-	-	NA
Pine Marten 1	Mustellids	Heart	+	-	-	-	+	*S. lutrae*
Pine Marten 2	Mustellids	Heart	+	-	-	-	+	*S. lutrae*
M58	Mink	Neck	-	-	-	-	-	NA
		Tongue	+	-	-	-	+	*S. lutrae*
H1	Horse	Muscle	+	-	-	-	+	*S. fayeri*
H2	Horse	Muscle	+	-	-	-	+	*S. fayeri*
H3	Horse	Muscle	+	-	-	-	+	*S. fayeri*
H4	Horse	Muscle	+	-	-	-	+	*S. fayeri*
H5	Horse	Muscle	+	-	-	-	+	*S. fayeri*
H6	Horse	Muscle	+	-	-	-	+	*S. fayeri*
H7	Horse	Muscle	+	-	-	-	+	*S. fayeri*
H8	Horse	Muscle	+	-	-	-	+	*S. fayeri*
H9	Horse	Muscle	+	-	-	-	+	*S. fayeri*
H10	Horse	Muscle	-	-	-	-	-	NA

H11	Horse	Muscle	-	-	-	-	-	NA
H12	Horse	Muscle	-	-	-	-	-	NA
H13	Horse	Muscle	-	-	-	-	-	NA
H14	Horse	Muscle	+	-	-	-	+	*S. fayeri*
Whale 1	Whale	Muscle	-	-	-	-	-	NA
Ba010	Badgers	Brain	+	+	-	+	-	*T. gondii*
Ba011	Badgers	Brain	-	-	-	-	-	NA
		Neck muscle	+	-	-	-	+	*S. lutrae*
Ba012	Badgers	Brain	-	-	-	-	-	NA
Ba013	Badgers	Brain	-	-	-	-	-	NA
Ba014	Badgers	Heart	+	+	-	+	-	*T. gondii*
		Kidney	+	+	-	+	-	*T. gondii*
Ba016	Badgers	Spleen	+	+	-	+	-	*T. gondii*
		Leg muscle	+	+	-	+	-	*T. gondii*
Ba017	Badgers	Neck muscle	+	+	+	+	-	*N. caninum/ T. gondii**
Ba018	Badgers	Lung	+	+	-	+	-	*T. gondii*
		Neck muscle	+	+	+	-	+	*N. caninum/ S. lutrae**
		Leg muscle	+	+	-	+	-	*T. gondii*
Ba019	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
Ba020	Badgers	Neck muscle	+	+	-	+	-	*T. gondii*
		Lymph node	+	+	-	+	-	*T. gondii*
Ba021	Badgers	Brain	+	+	-	+	-	*T. gondii*
		Neck muscle	+	-	-	-	+	*S. lutrae*
Ba022	Badgers	Brain	+	+	-	+	-	*T. gondii*
		Spinal cord	+	+	-	+	-	*T. gondii*
		Neck muscle	+	-	-	-	+	*S. lutrae*
		Spleen	+	+	-	+	-	*T. gondii*
Ba023	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Spleen	+	+	-	+	-	*T. gondii*
Ba024	Badgers	Brain	-	-	-	-	-	NA
		Spinal cord	-	-	-	-	-	NA
		Neck muscle	-	-	-	-	-	NA
Ba025	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
Ba026	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
Ba027	Badgers	Neck muscle	-	-	-	-	-	NA
Ba028	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
Ba029	Badgers	Neck muscle	+	+	-	+	+	*T. gondii/ S. lutrae**
Ba030	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
Ba031	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Heart	-	-	-	-	-	NA
Ba032	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*

		Heart	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba033	Badgers	Neck muscle	-	-	-	-	-	NA
		Tounge	+	-	-	-	+	*S. lutrae*
Ba034	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba035	Badgers	Neck muscle	-	-	-	-	-	NA
		Tounge	-	-	-	-	-	NA
Ba036	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba037	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba038	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba039	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba040	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba041	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba042	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba043	Badgers	Neck muscle	+	+	+	-	+	*N. caninum/ S. lutrae**
		Tounge	+	-	-	-	+	*S. lutrae*
Ba044	Badgers	Neck muscle	-	-	-	-	-	*NA*
		Tongue	+	-	-	-	+	*S. lutrae*
Ba045	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba046	Badgers	Neck muscle	-	-	-	-	-	NA
		Tounge	+	-	-	-	+	*S. lutrae*
Ba047	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba048	Badgers	Heart	+	+	-	+	-	*T. gondii*
		Tounge	-	-	-	-	-	NA
		Neck muscle	-	-	-	-	-	NA
Ba049	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba050	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
		Heart	+	+	-	+	-	*T. gondii*
Ba051	Badgers	Tounge	+	-	-	-	+	*S. lutrae*
		Neck muscle	-	-	-	+	-	NA

Ba052	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	-	-	-	-	-	NA
		Blood	+	+	-	+	-	*T. gondii*
Ba053	Badgers	Neck muscle	-	-	-	-	-	NA
		Tounge	-	-	-	-	-	NA
Ba054	Badgers	Neck muscle	-	-	-	-	-	NA
		Tounge	-	-	-	-	-	NA
Ba055	Badgers	Neck muscle	-	-	-	-	-	NA
		Tounge	-	-	-	-	-	NA
Ba056	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba057	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	+	-	+	+	*T. gondii/ S. lutrae**
Ba058	Badgers	Neck muscle	+	-	-	-	+	*S. lutrae*
		Tounge	+	-	-	-	+	*S. lutrae*
Ba059	Badgers	Neck	+	-	-	-	+	*S. lutrae*
Ba060	Badgers	Tounge	+	+	-	+	+	*T. gondii/ S. lutrae**
		Neck muscle	+	-	-	-	+	*S. lutrae*
		Spinal cord	+	+	-	+	-	*T. gondii*
Ba061	Badgers	Tounge	+	-	-	-	+	*S. lutrae*
		Neck muscle	+	-	-	-	+	*S. lutrae*
		Spinal cord	-	-	-	-	-	NA
		Liver	+	+	+	-	-	*N. caninum*
Ba062	Badgers	Heart	+	+	-	+	-	*T. gondii*
		Tounge	+	-	-	-	+	*S. lutrae*
		Neck muscle	-	-	-	-	-	NA
Ba063	Badgers	Tonge	+	-	-	-	+	*S. lutrae*
		Spinal cord	-	-	-	-	-	NA
		Neck muscle	+	-	-	-	+	*S. lutrae*
Ba064	Badgers	Liver	+	+	+	-	-	*N. caninum*
		Leg muscle	-	-	-	-	-	NA
		Neck muscle	-	-	-	-	-	NA

Legend. NA= not applicable, += positive results, -= negative results. 18S PAN represents primers (NTS-18S-F1 + NTS-18S-R1 and NTS-18S-F2 + NTS-18S-R2), 18S Neo Toxo represents (NTS-18S-F1 + NTS-18S-R1 and NTH-18S-F2 + NTH-18S-R2), ITS1 Neo represents (NTH-ITS1-F+NTH-ITS1-R and NEO-NP1 + NEO-NP2, ITS1 Toxorepresents (NTH-ITS1-F+NTH-ITS1-R and TOXO-NP1 + TOXO-NP2), 18S Sarco represents (NTS-18S-F1 + NTS-18S-R1 AND S-18S-G1, S-18S-G2, S-18S-G3, S-18S-G4, S-18S-G5, S-18S-G6 and S-18S-G9), * represents mixed infections.

APPENDIX TABLE V DE-WAXING PROGRAM USING A SERIES OF REAGENTS.

Solution	Immersion time (mins)
Xylene	5 minutes
Xylene	3 minutes
Methylated spirit Industrial 74 O.P (IMS)	2 minutes
IMS 50:50 IMS: Water	2 minutes
Water	1 minutes 30 seconds

APPENDIX TABLE VI. A SOURCE OF CONTROL TISSUE BLOCKS USED FOR THE IHC STUDY

Animal number	Information and source of samples
Cat brain 1/2	Clinical case from Royal Veterinary College, London (Dr. Henny Martineau)
MP14/630	Experimental case with BrI *T. gondii* strain from Agência Paulista de Tecnologia dos Agronegócios (Dr Daniela Pontes Chiebao)
MP12/929	Clinical case from Royal Veterinary College, London (Dr Stephen Cahalan)
11838	Clinical case from Pisa University, Italy (Prof. Carlo Cantile)
11820	
MP06/0228	Field case from Moredun Research Institute
MP15/0847	Field case from Moredun Research Institute
MP15/0848	Field case from Moredun Research Institute

APPENDIX TABLE VI.B DETAILS OF CONTROL TISSUE BLOCKS USED FOR THE IHC STUDY

Animal number	Host species	Previous results from database						Recent analysis using polyclonal serum							
		Tissue	Ab tested	Block n° tested	IHC results	ITS1 PCR	Sequencing results	Block n° tested	Neo NcSAG1	Neo NcSRS2	Toxo TgSRS2	Thermo Toxo	IHC Results	18S and ITS1 PCR	Sequencing results
Cat brain	Cat	Brain	anti-Toxo	1	+ *T. gondii*	+	*T. gondii*	1	-	-	+	+	*T. gondii*	+	*T. gondii*
				2	+ *T. gondii*	+	*T. gondii*	2	-	-	+	+	*T. gondii*	+	*T. gondii*
MP14/630	Mouse	Liver	anti-Toxo	1	+ *T. gondii*	+	*T. gondii*	1	-	-	+	+	*T. gondii*	+	*T. gondii*
MP12/929	Dog	Brain	anti-Neo	2	+ *N. caninum*	+	*N. caninum*	2	ND	+	-	ND	*N. caninum*	+	*N. caninum*
				4	+ *N. caninum*	NL	NA	4	ND	+	-	ND	*N. caninum*	+	*N. caninum*
				9	+ *N. caninum*	NL	NA	9	ND	+	-	ND	*N. caninum*	+	*N. caninum*
				12	+ *N. caninum*	NL	NA	12	ND	+	-	ND	*N. caninum*	+	*N. caninum*
				14	+ *N. caninum*	NL	NA	14	ND	+	-	ND	*N. caninum*	+	*N. caninum*
11838	Dog	Brain	anti-Neo	6	+ *N. caninum*	NL	NA	6	ND	+	-	ND	*N. caninum*	+	*N. caninum*
				11	+ *N. caninum*	NL	NA	11	ND	+	-	ND	*N. caninum*	+	*N. caninum*
11820	Dog	Brain	anti-Neo	1	+ *N. caninum*	NL	NA	1	ND	+	-	ND	*N. caninum*	+	*N. caninum*
				6	+ *N. caninum*	NL	NA	6	ND	+	-	ND	*N. caninum*	+	*N. caninum*
				8	+ *N. caninum*	+	*N. caninum*	8	ND	+	-	ND	*N. caninum*	+	*N. caninum*
MP06/0228	Ovine	Heart	anti-Sarco	NA	+ *S. tenella*	NL	NA	1	-	-	-	-	-	+	*S.tenella**
MP15/0847	Ovine	NA	NA	NA	NA	NA	NA	1	-	-	-	-	-	+	*S.tenella**
MP15/0848	Ovine	NA	NA	NA	NA	NA	NA	1	-	-	-	-	-	+	*S.tenella**

Legend. Na= not applicable, NL= information not listed, ND= not done, += positive, -= negative results, * samples only tested with 18S PCR

APPENDIX TABLE VII. RESULTS OF THE POLYCLONAL SERA BY IMMUNOHISTOCHEMISTRY USING CASES FROM MOREDUN SURVEILLANCE ARCHIVE

Animal identifier	Host species	Information availabe from the surveillance database				Recent IHC analysis using polyclonal serum						PCR analysis	
		Tissue	Ab tested	Block n° tested	Results	Tissue	Block n° tested	Anti-Neo NcSAG1	Anti-Neo NcSRS2	Anti-Toxo TgSRS2	Anti-Toxo (com.)	18S and ITS1 PCR	Sequencing results
MP08/727	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	5	+	+	-	-	ND	NA
MP08/1015	Bovine	Mixed	anti-Neo	NL	Inconc.	Brain	3	-	-	-	-	+	*N. caninum*
MP08/1022	Bovine	Brain	anti-Neo	NL	-	Brain	2	-	-	-	-	+	*N. caninum*
MP08/1103	Bovine	Mixed	anti-Neo	NL	-	Brain	5	+	+	-	-	+	*N. caninum*
MP08/1172	Bovine	Brain	anti-Neo	NL	Inconc.	Brain	11	-	-	-	-	+	*N. caninum*
MP08/1211	Bovine	Brain	anti-Neo	NL	-	Brain	2	-	-	-	-	+	*N. caninum*
MP08/1212A	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	12	-	-	-	-	-	NA
MP08/286A	Ovine	Placenta	anti-Toxo	NL	-	Placenta	1	ND	-	-	-	-	NA
MP08/286B	Ovine	Placenta	anti-Toxo	NL	-	Placenta	2	-	-	-	+	ND	NA
MP08/386	Bovine	Placenta	anti-Toxo	NL	-	Placenta	3	ND	-	-	-	-	NA
MP08/557	Bovine	Brain	anti-Neo	NL	-	Brain	1	-	-	-	-	-	NA
MP08/623	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	9	ND	-	-	-	ND	NA
MP08/640	Bovine	Brain	anti-Neo	NL	-	Brain	1	-	-	-	-	ND	NA
MP08/941	Bovine	Brain	anti-Neo	NL	-	Brain	2	-	-	-	-	-	NA
MP08/961	Bovine	Brain	anti-Neo	NL	-	Brain	4	-	+	-	-	+	*N. caninum*
MP09/1153	Bovine	Brain	anti-Neo	NL	-	Brain	4	-	-	-	-	-	NA
MP09/118A	Ovine	Brain	anti-Toxo	NL	-	Brain	4	-	-	-	-	+	*T. gondii*
MP09/118C	Ovine	Brain	anti-Toxo	NL	-	Brain	2	-	-	-	-	+	*T. gondii*
MP09/121	Bovine	Mixed	anti-Neo	NL	+ *N. caninum*	Brain	3	-	+	-	-	+	*N. caninum/ S. cruzi*
MP09/122	Bovine	Placenta	anti-Neo	NL	-	Placenta	5	-	-	-	-	+	*N. caninum*
MP09/1249A	Bovine	Mixed	anti-Neo	NL	-	Brain	6	-	-	-	-	-	NA
MP09/1264A	Bovine	Mixed	anti-Neo	NL	+ *N. caninum*	Brain	6	ND	+	-	-	ND	NA
MP09/1264B	Bovine	Mixed	anti-Neo	NL	+ *N. caninum*	Brain	1	ND	ND	-	-	-	NA
MP09/1264C	Bovine	Mixed	anti-Neo	NL	-	Brain	1	+	+	-	-	-	NA
MP09/1268	Bovine	Mixed	anti-Neo	NL	-	Brain	1	-	-	ND	ND	ND	NA
MP09/131	Ovine	Placenta	anti-Toxo	NL	Inconc.	Placenta	2	-	-	-	-	-	NA
MP09/274	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	3	-	-	-	-	-	NA
MP09/286	Ovine	Placenta	anti-Toxo	NL	+ *T. gondii*	Placenta	1	-	-	+	+	+	*T. gondii*
MP09/819	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	4	-	-	-	-	-	NA

MP09/831	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	3	-	-	-	-	-	NA
MP10/1059	Bovine	Brain	anti-Neo	NL	-	Brain	3	-	-	-	-	-	NA
MP10/35	Bovine	Mixed	anti-Neo	NL	+ *N. caninum*	Brain	4	-	-	-	-	ND	NA
MP10/471A	Bovine	Brain	anti-Neo	NL	-	Brain	2	-	-	-	-	ND	NA
MP10/838	Bovine	Brain	anti-Neo	NL	-	Brain	2	-	-	-	-	-	NA
MP10/890	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	1	-	-	-	-	-	NA
MP11/1061	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	3	-	-	-	-	-	NA
MP11/1062	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	7	-	-	-	-	-	NA
MP11/1077	Bovine	Mixed	anti-Neo	NL	-	Brain	2	-	-	-	-	-	NA
MP11/1081	Bovine	Brain	anti-Neo	NL	-	Brain	2	-	-	-	-	ND	NA
MP11/225	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	2	+	+	-	-	ND	NA
MP11/276	Bovine	Brain	anti-Neo	NL	-	Brain	4	-	-	-	-	-	NA
MP11/32	Bovine	Brain	anti-Neo	NL	-	Brain	2	-	-	-	-	-	NA
MP11/543	Bovine	Brain	anti-Neo	NL	-	Brain	5	-	-	-	-	-	NA
MP11/546	Bovine	Brain	anti-Neo	NL	-	Brain	1	-	-	-	-	-	NA
MP11/547	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	1	-	-	-	-	-	NA
MP11/613	Bovine	Brain	anti-Neo	NL	-	Brain	2	-	-	-	-	-	NA
MP11/653	Bovine	Heart	anti-Neo	NL	-	Heart	4	-	-	-	-	-	NA
MP11/807	Bovine	Brain	anti-Neo	NL	-	Brain	5	+	+	-	-	+	*N. caninum*
MP12/0785	Bovine	Mixed	anti-Neo	3,4,6,7,10	Inconc.	Brain	6	-	-	-	-	-	NA
MP12/1241	Bovine	Brain	anti-Neo	3,6,7,8	+ *N. caninum*	Brain	6	-	-	-	-	ND	NA
MP12/1275B	Bovine	Brain	anti-Neo	6,7,9	+ *N. caninum*	Brain	6	+	+	-	-	ND	NA
MP12/1316	Bovine	Mixed	anti-Neo	NL	-	Brain	8	-	-	-	-	-	NA
MP12/1330	Ovine	Mixed	anti-Toxo	NL	NL	Heart	15	-	-	-	-	ND	NA
MP12/1339	Bovine	Brain	anti-Neo	NL	-	Brain	2	+	+	-	-	ND	NA
MP12/1364	Bovine	Brain	anti-Neo	1,2,3	-	Brain	3	-	-	-	-	-	NA
MP12/185	Bovine	Mixed	anti-Neo	NL	+ *N. caninum*	Brain	4	+	+	-	-	ND	NA
MP12/215	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	5	-	+	-	-	ND	NA
MP12/220	Bovine	Mixed	anti-Neo	NL	+ *N. caninum*	Brain	7	+	+	-	-	ND	NA
MP12/287	Ovine	Mixed	anti-Toxo	NL	+ *T. gondii*	Brain	9	-	-	-	-	+	*T. gondii*
MP12/486B	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	3	-	+	-	-	+	*N. caninum*
MP12/593	Bovine	Placenta	anti-Neo	NL	+ *N. caninum*	Placenta	3	-	-	-	-	-	NA
MP13/0825	Bovine	Brain	anti-Neo	4,7,8	+ *N. caninum*	Brain	7	+	+	-	-	-	NA
MP13/110	Bovine	Mixed	anti-Neo	NL	+ *N. caninum*	Brain	4	-	-	-	-	-	NA
MP13/267A	Ovine	Brain	anti-Toxo	2 3	-	Brain	2	-	-	-	-	-	NA
MP13/267B	Ovine	Brain	anti-Toxo	4	-	Brain	4	+	+	-	-	+	*T. gondii*
MP13/267C	Ovine	Placenta	anti-Toxo	1	-	Placenta	1	-	-	-	-	-	NA
MP13/270	Bovine	Brain	anti-Neo	3,5,6,7	+ *N. caninum*	Brain	5	-	+	-	-	ND	NA

MP13/677	Bovine	Brain	anti-Toxo	4,10,12,15	-	Brain	15	-	-	-	-	-	NA
MP13/804	Bovine	Brain	anti-Neo	7,8	-	Brain	7	-	-	-	-	ND	NA
MP13/808	Bovine	Brain	anti-Neo	4,5	Inconc.	Brain	4	-	-	-	-	ND	NA
MP13/817	Bovine	Brain	anti-Neo	4,5	-	Brain	4	-	ND	ND	-	ND	NA
MP13/931	Bovine	Brain	anti-Neo	8,12	+ *N. caninum*	Brain	8	-	-	-	-	ND	NA
MP13/955	Bovine	Placenta	anti-Neo	NL	-	Placenta	2	-	-	-	-	ND	NA
MP13/984	Bovine	Mixed	anti-Neo	7,8,10	-	Brain	7	+	+	-	-	ND	NA
MP14/0162	Ovine	Brain	anti-Toxo	8, 9	-	Brain	8	-	-	-	-	-	NA
MP14/0231	Ovine	Brain	anti-Toxo	1, 3	NL	Brain	1	-	ND	-	-	-	NA
MP14/0706	Bovine	Brain	anti-Neo	8,9,10	+ *N. caninum*	Brain	8	-	-	-	-	-	NA
MP14/0707	Bovine	Brain	anti-Neo	2,3,6	+ *N. caninum*	Brain	3	-	-	-	-	+	*T. gondii**
MP14/0718A	Bovine	Brain	anti-Neo	6,8	-	Brain	8	-	-	-	-	ND	NA
MP14/0736	Bovine	Brain	anti-Neo	NL	+ *N. caninum*	Brain	7	-	+	-	-	+	NA
MP14/0745	Ovine	Brain	anti-Sarco	2,6,7	-	Brain	2	-	-	-	-	-	NA
MP14/0797	Bovine	Brain	anti-Neo	1,2,15,16,17	+ *N. caninum*	Brain	16	-	-	-	-	-	NA
MP14/0958	Bovine	Brain	anti-Neo	7, 9	-	Brain	7	-	-	-	-	+	*T. gondii**
MP15/0006	Bovine	Mixed	anti-Neo	NL	Inconc.	Brain	9	-	-	-	-	+	*N. caninum*
MP15/0014	Bovine	Heart	anti-Neo	NL	Inconc.	Heart	23	-	-	ND	-	-	NA
MP15/0202	Ovine	Heart	anti-Toxo	NL	NL	Heart	3	-	-	-	-	-	NA
MP15/0775	Bovine	Mixed	anti-Neo	9,12,14	+ *N. caninum*	Brain	9	+	+	-	-	ND	NA
MP15/0865-A	Ovine	Heart	anti-Sarco	3	+ *Sarcocystis*	Heart	3	-	-	-	-	+	*S.gigantea*
MP15/0866-B	Ovine	Heart	anti-Sarco	1	+ *Sarcocystis*	Heart	1	-	-	-	-	+	*S.gigantea*
MP15/0867-B	Ovine	Heart	anti-Sarco	5	+ *Sarcocystis*	Heart	5	-	-	-	-	+	*S.gigantea*
MP15/0868-B	Ovine	Heart	anti-Sarco	6	+ *Sarcocystis*	Heart	6	-	-	-	-	+	*S.gigantea*
MP15/0901	Bovine	Brain	anti-Neo	2,7,10,11	-	Brain	2	+	+	-	-	+	*N. caninum*
MP15/0910	Bovine	Brain	anti-Neo	6,7,10	+ *N. caninum*	Brain	10	+	+	-	-	ND	NA
MP15/0911	Bovine	Brain	anti-Neo	5,6,12	+ *N. caninum*	Brain	5	-	-	-	-	ND	NA
MP15/0913	Bovine	Brain	anti-Neo	7,8,9	+ *N. caninum*	Brain	8	+	+	-	-	ND	NA
MP15/0948	Bovine	Brain	anti-Neo	1,8,10,16	+ *N. caninum*	Brain	8	-	-	-	-	-	NA
MP16/0211	Ovine	Brain	anti-Toxo	1,2,3,4,5	NL	Brain	8	-	-	-	-	+	*S.tenella*
MP16/0370B	Ovine	Brain	anti-Toxo	1,2,5,8	+ *T. gondii*	Brain	5	-	-	+	+	+	*T. gondii*
MP16/0953	Bovine	Brain	anti-Neo	1, 4,	-	Brain	4	-	-	-	-	ND	NA
MP17/0012	Bovine	Brain	anti-Neo	NL	-	Brain	2	-	-	-	-	-	NA
MP17/0013	Bovine	Brain	anti-Neo	3,8,13,14	-	Brain	3	-	-	-	-	-	NA

MP17/0021A	Bovine	Brain	anti-Neo	4,6	-	Brain	6	-	-	-	-	-	NA
MP17/0023	Bovine	Brain	anti-Neo	2,3,4	+ *N. caninum*	Brain	4	-	-	-	-	-	NA
MP17/0062	Ovine	Brain	anti-Toxo	8, 9	-	Brain	3	-	ND	ND	ND	-	NA
MP17/0261	Ovine	Brain	anti-Toxo	NL	NL	Brain	1	-	-	-	-	+	*T. gondii*
MP17/0374	Ovine	Brain	anti-Toxo	NL	NL	Brain	1	-	-	-	-	ND	NA
MP17/0562	Ovine	Brain	anti-Toxo	NL	NL	Brain	1	-	-	-	-	+	*S.tenella*
MP17/0630	Ovine	Brain	anti-Toxo	NL	NL	Brain	2	-	-	-	-	-	NA
MP17/0631	Bovine	Brain	NL	NL	NL	Brain	5	-	-	-	-	ND	NA
MP17/689	Bovine	Brain	anti-Neo	NL	NL	Brain	15	-	-	-	-	+	*T. gondii**
MP17/732	Bovine	Brain	anti-Neo	NL	NL	Brain	1	-	-	-	-	-	NA

Legend. NA= not applicable, NL= information not listed, ND= not done (due to technical reason some samples were not done), += positive results, -= negative results, Inconc.= inconclusive results. Anti-Neo =polyclonal rabbit anti-Neospora (NC1), anti-Toxo= polyclonal rabbit anti-Toxoplasma (M1/M2-669) anti-Sarco= polyclonal rabbit anti-Sarcocystis (9953311), anti-Neo NcSAG1 =polyclonal rabbit anti-Neospora-NcSAG1, anti-Neo NcSRS2= polyclonal rabbit anti-Neospora-NcSRS2, anti-Toxo TgSRS2= polyclonal rabbit anti-Toxoplasma-TgSRS2, anti-Toxo (com.)= rabbit anti-T. gondii IgG antibody (Thermofisher). T. gondii* show potential false posivites due to contamination of the PCR sample. ITS1 PCR represent ITS1 Neo (NTH-ITS1-F+NTH-ITS1-R and NEO-NP1 + NEO-NP2 and ITS1 Toxo (NTH-ITS1-F+NTH-ITS1-R and TOXO-NP1 + TOXO-NP2), 18S PCR represents 18S Pan primers (NTS-18S-F1 + NTS-18S-R1 AND NTS-18S-F2 + NTS-18S-R2), 18 NEO/ TOXO (NTS-18S-F1 + NTS-18S-R1 AND NTH-18S-F2 + NTH-18S-R2), and 18S Sarco (NTS-18S-F1 + NTS18S-R1 and S-18S-G1, S-18S-G2, S-18S-G3, S-18S-G4, S-18S-G5 and S-18S-G6).

Appendix II. Receipts of buffer and solutions

Phosphate buffer saline (PBS)

Potassium dihydrogen phosphate Chloride (Fisher Scientific®) 2.4 g
Potassium Chloride (Fisher Scientific®)
2.0 g
Sodium dihydrogen phosphate heptahydrate Chloride (FisherScientific®) 26.8 g
1x BPS is diluted 1:10
10x was made up in 800mls of dH_2O

PBS Tween 20 (PBST)

PBS containing 0.05% Tween 20
PBS 1X 1000 ml
Tween 20 (Sigma-Aldrich®) 500 µl
Final solution was autoclaved

Tris-buffered saline wash buffer (TBS)

1M Tris HCl stock 125 ml
5M NaCl 75 ml
Purified water 2300 ml
Mix and adjust pH 7.6. Add purified water to give final volume of 2.5 litre

25% Normal Goat serum- per 1 slide

Normal goat serum (Abcam) 1 µl
TBS 3 µl

50X TAE Buffer

Tris (Sigma-Aldrich®) 242.0 g
0.5M EDTA (Sigma-Aldrich®) 100 ml
Glacial acetic acid (CH_3CO_2H) (Sigma-Aldrich®) 57.1 ml
Make up to 1 litre of distilled water

1X TAE Buffer

50X TAE 20 ml
Distilled water 980 ml

50mg/ml Ampicillin

Ampicillin (sodium salt) (Sigma-Aldrich®) 500 mg
Distilled Water 10 ml
Mix to dissolve and filter sterilise 0.45nm. Aliquot into 200µl

50mg/ml X-Galactosidase

X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) in N,N-dimethylformamide (Promega)
Recommended use with pGEMT easy 20µl of 50mg/ml X Gal per plate

0.1ml IPTG

IPTG (Isopropyl-β-D-thiogalactopyranoside) (Promega) 200 mg
Distilled water 1 ml
Mix, filter sterilise 0.45nm, and dispense into aliquots.
Recommended use with pGEMT easy 100µl of 0.1M IPTG per plate

Citrate Buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0):

Citric acid (anhydrous) (Fisher Scientific®)	2.1 g
Distilled water	1000 ml

Mix to dissolve. Adjust pH to 6.0 with 1M NaOH (28mls)

Proteas Solution (0.1% in TBS)

Protease (Fisher Scientific®)	0.25 g
TBS	250 ml

Albumin solution

Albumin (Fisher Scientific®)	5 mg
Glycerol (Sigma-Aldrich®)	50 ml
dH2O	50 ml

Mayer's Haematoxylin

Mayer's Haematoxylin (CellPath Ltd., Powis, UK)	1.0 g	
Sodium iodate ($NaIO_3$) (Fisher Scientific ®)	0.2 g	
Potassium aluminium sulphate ($KAI(SO_4)_2$) (Fisher scientific ®)		50.0 g
Chloral hydrate ($C_2H_3Cl_3O_2$) (Fisher scientific ®)	50.0 g	
Citric acid ($C_6H_8O_7$) (Fisher scientific ®)	1.0 g	
Purified water		1000 ml

Dissolve the first 3 ingredients in purified water mix and leave overnight at room temperature. Add the remaining ingredients, boil for 5 minutes, cool and filter.

Scott's tap water substitute (STWS)

Sodium bicarbonate ($NaHCO_3$) (Fisher Scientific®)	3.5 g	
Magnesium sulphate ($MgSO_4$) (Fisher Scientific®)	20.0 g	
Tap water		1000 ml

Protein purification buffers

Binding buffer (300mls)

200mM Monosodium Phosphate- NaH_2PO_4 (Sigma-Aldrich®)		5.7 ml
200mM Disodium Phosphate-Na_2HPO_4 (Sigma-Aldrich®)	24.3 ml	
8 M Urea (Promega)		144.14 g
500mM Sodium Chloride-NaCl (Fisher Scientific®)	8.7 g	

Use sterile water, 1st add 150mls than take volume to 300mls

Potentially add 10mM Imidazole. Warm up H2O before denaturing buffer to ease Urea dissolution.

Sonication buffer

50mM NaH_2PO_4 (from 1M stock) (Fisher scientific ®)		5 ml
300mM NaCl (from 1 M stock) (Fisher scientific ®)	30.3 ml	
10mM Imidazole (from 1M stock) (Sigma-Aldrich®)	0.5 ml	
0.25% Tween 20	0.25 ml	
dH2O		63.9 ml

Make up to 100mls

Equilibration Buffer

300mM NaCl (10x PBS) (as above)	10 ml	
10mM Imidazole (from 1M stock) (Fisher Scientific®)		1 ml

dH20		50 ml
8M UREA (un-soluble condition only) (As above)	48.1 g	

Add reagents together dissolve UREA first in PBS and water. Adjust pH to 7.4 and top up with water to make 100ml.

Wash Buffer

300mM NaCl (10x PBS) (as above)	10 ml	
25mM Imidazole (from 1M stock) (as above)		2.5 ml
50mM Imidazole (from 1M stock) (as above)		5 ml
dH20		50 ml
8M UREA (un-soluble condition only) (as above)	48.1 g	

Add reagents together dissolve UREA first in PBS and water. Adjust pH to 7.4 and top up with water to make 100mls

Elution Buffer

300mM NaCl (10x PBS) (as above)	10 ml	
250mM Imidazole (from 1M stock) (as above)		25 ml
dH20		40 ml
8M UREA (un-soluble condition only) (as above)	48.1 g	

Add reagents together dissolve UREA first in PBS and water.
Adjust pH to 7.4 and top up with water to make 100mls

MES Buffer (200mls)

20mM Ethanosulfonic acid 2-(N-morpholie) (Sigma-Aldrich®)		0.78 g
100mM NaCl (pH 5) (as above)		1.17g

Western blot buffers

Wash buffer

Can be made up 1 week before. Make up to 2 litres.

Tween 80 (Sigma-Aldrich®)		10 ml
NaCl (as above)		58.44 g
10x PBS	200 ml	
dH2O make up to 2l		

Block Buffer

Dissolve Marvel using magnetic stirrer. Filter Marvel using filter paper.

4% Marvel (Fisher Scientific®)		10 g
1x PBS		250 mls

Diluent Buffer (2% Marvel)

Made up from 4% Marvel (10ml) in 20ml wash buffer.

4% Marvel (as above)		5 ml
Wash buffer		5 ml

Transfer buffer- non-reduced

NuPAGE® Transfer Buffer (20x) (Sigma-Aldrich®)	50 ml	
Methanol (20% gels) (Fisher Scientific®)	200 ml	
dH2O		750 ml

NuPAGE Transfer buffer with 10% (100mls) methanol provides transfer on a single gel, while 20% (200mls) ensure sufficient transfer for two gels.

10X PCR Buffer solutions

2mM Tris HCL (p.H. 8.8)

Tris (as above)	60.55 g
dH2O	200 ml

Adjust to pH 8.8. with HCL. Make µp to 250mls with dH2O

1M Ammonium Sulphate

Ammonium Sulphate (Sigma-Aldrich®)	33.0 g
dH2O	250 ml

0.5M EDTA stock to 10mM

0.5M EDTA (as above)	5 ml
dH2O	245 ml

BSA 10mg/ml

BSA (Fisher Scientific®)	200 mg
dH2O	20 ml

1 MgCl

$MgCl_2$ (Fisher Scientific®)	40.66 g
dH2O	200 ml

Made up to volume of 10mls and aliquoted into 1mls. Stock solutions are autoclaved under UV light for 30 minutes prior to making 10X buffer

Reagents	Stock conc.	Volume	Final conc.	Conc. In reactions
Tris-HCl	2M	2250µl	450mM	45mM
Ammonium Sulphate	1M	1100µl	110mM	11mM
$MgCl_2$	1M	450µl	45µM/	4.5mM
EDTA (pH 8)	1mM	44µl	44µM/	4.4µM
BSA	10mg/ml	1130µl	1.13mg/ml	
dATP	100mM 1000µl	10mM	1.0mM	
dCTP	100mM 1000µl	10mM	1.0mM	
dGTP	100mM 1000µl	10mM	1.0mM	
dTTP	100mM 1000µl	10mM	1.0mM	
dH2O	1026µl			

Digestate fluid extraction of *Sarcocystis* merozoites from Sheep hearts

Fluid was added to mince to 5:1 (fluid: mince)

If 4.12.8 g of mince was obtained (5 x 412.8 =2.064) -	2.06L of Digestate
0.26% pepsin (0.26 x 2.064= 0.54) -	5.4g of pepsin in 2 L of PBS
0.7% HCl in PBS (0.7 x 2.064= 1.44) -	14ml HCl in 2 L of PBS
30% Percoll (Sigma-Aldrich®) of 200mls PBS	60ml Percoll in 140mls PBS
90% Percoll of 30ml PBS	27ml Percoll in 3mls PBS

Appendix III. Publications and Presentations

1.1. Peer reviewed publications- Published (2)

Lepore, T., Bartley, P., Chianini, F., Macrae, A., Innes, E., & Katzer, F. (2017). Molecular detection of *Sarcocystis* lutrae in the European badger (*Meles meles*) in Scotland. *Parasitology,* **144,** 1-7. doi:10.1017/S0031182017000762

Bartley, P.M., Chia, S. L., Gilray, J., Shawh, J., **Lepore, T**., Gunn_Moore, D., Innes, E. A., and Katzer, F. Detection of potentially human infectious assemblages of *Giardia duodenalis* in wild rodents in Scotland.

1.2. Peer reviewed publications- In preparation (2)

Lepore, T., Cantón, G.J., Cantile, C., Martineau, H. M., Palarea-Albaladejo, J., Katzer, Macrae, A., Innes, E., &. F., Chianini, F. Genus-specific detection of *Neospora caninum* and *Toxoplasma gondii* using antibodies raised against protozoan recombinant proteins that can be used for the diagnosis of ruminant abortion cases.

Lepore, T., Chianini, F., Macrae, A., Innes, E., & Katzer, F. Discrimination of *Neospora caninum*, *Toxoplasma gondii* and *Sarcocystis* spp. in ruminants by PCR using fixed or fresh tissue samples.

1.3. Conference proceedings- Oral and poster presentations (22)

2018

T. Lepore. Use of antibodies developed during PhD to develop various assays to be used validate *Neospora* vaccine candidates. Moredun Research Institute, Midlothian, Scotland 16th October. Talk

T. Lepore, A.I. Macrae, F. Katzer, and F. Chianini. Specific Diagnostic tools for protozoan infection of ruminants. Zoetis. Moredun Research Institute, Midlothian, Scotland. 4th October. PhD seminar.

T. Lepore, A.I. Macrae, F. Katzer, and F. Chianini. Specific Diagnostic tools for protozoan infection of ruminants. Zoetis. Moredun Research Institute, Midlothian, Scotland. 25th September. PhD summary.

T. Lepore. Use of antibodies developed during PhD to develop various assays to be used validate *Neospora* vaccine candidates. Belgium, Louvain la Neuve. Zoetis. 9th August. Talk

T. Lepore. A.I. Macrae, F. Katzer, and F. Chianini. Development of genus-specific antibodies used for the diagnosis in abortion cases of ruminants. PhD Student Day at Moredun Research Institute, Edinburgh, Scotland. 19th April. Talk.

T. Lepore, A.I. Macrae, F. Katzer, and F. Chianini. *Development of genus-specific antibodies used for the diagnosis in abortion cases of ruminants*. PhD Student Day at Roslin Institute, Midlothian, Scotland. 18th April. Talk.

T. Lepore, A.I. Macrae, F. Katzer, and F. Chianini. Validation of the PCR and IHC techniques using ruminant diagnostic cases. Protozoal group. Moredun Research Institute, Edinburgh, Scotland. 13th February. Talk.

2017

T. Lepore, A.I. Macrae, F. Katzer, and F. Chianini. PhD on diagnostic tools for protozoan infection of ruminants. Glasgow Food Security student. Moredun Research Institute, Edinburgh, Scotland. 20th November. Talk.

T. Lepore, A.I. Macrae, F. Katzer, and F. Chianini. Specific Diagnostic tools for protozoan infection of ruminants. Zoetis. Roslin Institute, Midlothian, Scotland. 13th November. Poster.

T. Lepore, A.I. Macrae, F. Katzer, and F. Chianini. Genus-specific antibodies for the diagnosis of *Neospora caninum* and *Toxoplasma gondii*. International Apicowplexa conference, San Lorenzo de El Escorial Spain. 14th October. Poster.

T. Lepore, A.I. Macrae, F. Katzer, and F. Chianini. Genus-specific antibodies for the diagnosis of *Neospora caninum* and *Toxoplasma gondii*. Protozoal group. Moredun Research Institute, Edinburgh, Scotland. 12th June. Talk.

T. Lepore. A.I. Macrae, F. Katzer, and F. Chianini Specific diagnostic tool for protozoan infection of ruminants. PhD Student Day at Moredun Research Institute, Edinburgh, Scotland. 12th May. Talk.

T. Lepore, A.I. Macrae, F. Katzer, and F. Chianini. Genus-specific antibodies for the diagnosis of *Neospora caninum* and *Toxoplasma gondii*. PhD Student Day at Roslin Institute, Midlothian, Scotland. 10th May. Poster.

2016

T. Lepore, A.I. Macrae, F. Katzer, and F. Chianini. Specific Diagnostic tools for protozoan infection of ruminants. Zoetis. Roslin Institute, Midlothian, Scotland. 21st June. Talk.

T. Lepore, A.I. Macrae, F. Katzer and F. Chianini. Development of recombinant proteins. Protozoal group. Moredun Research Institute, Edinburgh, Scotland. 8th June. Talk.

T. Lepore. A.I. Macrae, F. Katzer, and F. Chianini. Polyclonal antiserum development using recombinant proteins. PhD Student Day at Moredun Research Institute, Edinburgh, Scotland. 6th May. Talk.

T. Lepore, PM. Bartley, F. Chianini. A.I. Macrae, EA Innes and F. Katzer. Detection of 18S DNA fragment identical to *Sarcocystis* lutrae in European badgers (*Meles meles*) in Scotland. PhD Student Day at Roslin Institute, Midlothian, Scotland. 27th April. Poster.

T. Lepore, PM. Bartley, F. Chianini. A.I. Macrae, EA Innes and F. Katzer. Detection of an 18S rDNA fragment in European badgers (*Meles meles*) in Scotland that shows 100% sequence identity to *Sarcocystis lutrae*. British Society of Parasitology Spring conference. 11th -13th April. Talk. Awarded a grant of £200.

2015

T. Lepore. Presentation of my PhD to HRH the Princess Royal. Moredun Research Institute, Edinburgh, Scotland. 16th October. Talk.

T. Lepore, A.I. Macrae, F. Katzer, and F. Chianini. Development of genus-specific molecular tools and development of recombinant proteins. Roslin Institute, Midlothian, Scotland. 2nd June. Talk.

T. Lepore. International Apicowplexa conference, Moredun Research Institute, Edinburgh, Scotland. 3rd- 5th July. Attendance and organising team.

T. Lepore. Science festival, Botanic garden, Edinburgh, Scotland. 13th April. Attendance and demonstrating.

1.4. Grants and awards

T. Lepore- British Society of Parasitology Spring conference. 2016. 11th -13th April. Awarded a £200 grant.

T. Lepore- University of Edinburgh Erasmus grant for the Industrial placement with Zoetis from May 15th to August 10th (£1089.00).

1.4. Publications

Molecular detection of *Sarcocystis lutrae* in the European badger (*Meles meles*) in Scotland

T. LEPORE[1], P. M. BARTLEY[1], F. CHIANINI[1], A. I. MACRAE[2], E. A. INNES[1] and F. KATZER[1]*

[1] *Moredun Research Institute, Pentlands Science Park, Bush Loan, Midlothian, EH26 0PZ, Scotland, UK*
[2] *Royal (Dick) School of Veterinary Studies and the Roslin Institute, Easter Bush Veterinary Centre, Roslin, Midlothian, EH25 9RG, Scotland, UK*

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SUMMARY

Neck samples from 54 badgers and 32 tongue samples of the same badgers (*Meles meles*), collected in the Lothians and Borders regions of Scotland, were tested using polymerase chain reactions (PCRs) directed against the 18S ribosomal DNA and the internal transcribed spacer (ITS1) region of protozoan parasites of the family Sarcocystidae. Positive results were obtained from 36/54 (67%) neck and 24/32 (75%) tongue samples using an 18S rDNA PCR. A 468 base pair consensus sequence that was generated from the 18S rDNA PCR amplicons (KX229728) showed 100% identity to *Sarcocystis lutrae*. The ITS1 PCR results revealed that 12/20 (60%) neck and 10/20 (50%) tongue samples were positive for Sarcocystidae DNA. A 1074 bp consensus sequence was generated from the ITS1 PCR amplicons (KX431307) and showed 100% identity to *S. lutrae*. Multiple sequence alignments and phylogenetic analysis support the finding that the rDNA found in badgers is identical to that of *S. lutrae*. This parasite has not been previously reported in badgers or in the UK. *Sarcocystis lutrae* has previously only been detected in tongue, skeletal muscle and diaphragm samples of the Eurasian otter (*Lutra lutra*) in Norway and potentially in the Arctic fox (*Vulpes lagopus*).

Key words: *Sarcocystis lutrae*, rDNA, Scotland, European badgers (*Meles meles*), 18S PCR, ITS1 PCR, phylogenetic analysis.

INTRODUCTION

Current knowledge suggests that Sarcocystosis is caused by 200 currently identified species of single-cell coccidian parasites in the phylum Apicomplexa, and genus *Sarcocystis*. These parasites infect a wide range of definitive and intermediate hosts, including carnivorous animals, domestic animals and humans (Dubey and Lindsay, 2006; Kaltungo and Musa, 2013; Dubey *et al*. 2015). All *Sarcocystis* parasites have an obligatory two host life cycle (some exception such as *Sarcocystis neurona* exist); asexual reproduction takes place in the intermediate host and sexual reproduction occurs in the intestine of the definitive host (Dubey and Lindsay, 2006). *Sarcocystis neurona* has a very broad intermediate host range and for example sporocysts from opossums can infect many hosts, of which some are natural intermediate hosts (in which sarcocysts are formed), while others are aberrant hosts (in which only schizonts are formed) (Dubey *et al*. 2001*a*). Transmission from definitive to intermediate host occur via the ingestion of oocysts/sporocysts from feces via contaminated food or water and transmission from intermediate to definitive host occur via the ingestion of sarcocysts, which are found in muscle tissue (Dubey and Lindsay, 2006; Gjerde and Josefsen, 2015). However, knowledge and understanding of all the life cycle stages of *Sarcocystis* species in wild carnivores is incomplete and needs to be researched in more detail to help us to better understand disease pathogenesis, symptomatology and impact of parasite diversity. More research is needed to determine the range of clinical manifestations of *Sarcocystis* infections in wild carnivores, as there is little information available about the signs and symptoms in these host species.

Only a few *Sarcocystis* species have been identified in wild carnivores of the family Mustelidae (Dubey *et al*. 2010). For example *S. neurona* has previously been identified in (Eurasian) otters (*Enhydra lutris*) (Dubey *et al*. 2001*b*, 2003; Miller *et al*. 2009; Wendte *et al*. 2010), while *S. lutrae* has been found in the Eurasian otter (*Lutra lutra*) in Norway (Gjerde and Josefsen, 2015). *Sarcocystis lutrae* has not been confirmed in another host species other than the Eurasian otter and potentially Arctic foxes (Gjerde and Schulze, 2014; Gjerde and Josefsen, 2015). Various *Sarcocystis* spp. including *Sarcocystis hofmanni*, *Sarcocystis melis*, *Sarcocystis cf. sebeki* and *Sarcocystis cf. gracilis*, have previously been recorded by light microscopy (LM) and transmission electron microscopy (TEM) in heart, thigh, loin, thorax and tongue samples in European

* Corresponding author: Moredun Research Institute, Pentlands Science Park, Bush Loan, Midlothian, EH26 0PZ, Scotland, UK. E-mail: Frank.Katzer@moredun.ac.uk

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badgers (*Meles meles*) from Berlin (Odening *et al*. 1994*a*, *b*). None of these *Sarcocystis* species in badgers have been identified in the UK. More recently, an unnamed species of *Sarcocystis* was recorded in the tongue, diaphragm and masseter muscle of Japanese badgers (*Meles anakuma*) using haematoxylin and eosin (H&E) staining (Kubo *et al*. 2009). To date LM of fresh muscle tissue and TEM have been examined to identify the *Sarcocystis* spp. found in badgers, meaning no DNA sequences are available for the *Sarcocystis* species previously identified in badgers. Only a few *Sarcocystis* species found in wild carnivores have been examined using molecular methods, these include species, such as *Sarcocystis arctica*, *Sarcocystis lutrae*, *Sarcocystis kalvikus*, and *Sarcocystis kitikmeotensis* (Dubey *et al*. 2015). Techniques, such as polymerase chain reactrion (PCR) and sequence analysis are more frequently used to identify *Sarcocystis* species (Gjerde and Josefsen, 2015). Polymorphisms in the 18S rDNA and internal transcribed spacer (ITS1) region may help with the speciation and discrimination of the different species within the *Sarcocystis* genus. The aim of this study was to determine the prevalence and species of *Sarcocystis* in muscle samples from European badgers (*M. meles*).

MATERIALS AND METHODS

Collection of samples

In total 54 European badger (*M. meles*) carcasses were collected from around the Lothians and Borders regions of Scotland, following fatal collisions with vehicles (badgers were collected with the knowledge and permission of Scottish Natural Heritage) (Bartley *et al*. 2013). Carcasses were stored at −20 °C prior to processing, full necropsies were performed when possible where samples of neck muscle, tongue, spleen, submandibular lymph node, liver, lung, brain, heart, blood and spinal cord were collected.

DNA extraction

DNA was extracted from muscle samples of 54 badgers. From those badgers, 54 neck samples and 32 tongue samples derived from the same animals were extracted. Approximately 1 g of each thawed tissue was transferred into a separate CK22 Precellys tissue homogenizer tube (Cepheid, Stretton Derbyshire, UK), containing 1 mL Nuclei Lysis Solution (Promega, Madison, WI, USA). Samples were homogenized for 2 × 50 s at 6500 rpm using a Precellys 24 tissue homogenizer (Depheid, Stretton Derbyshire, UK). 400 µL of each homogenised tissues were added to a further 900 µL of nuclei lysis solution and incubated at 55°C overnight. Samples were then processed using the Wizard® genomic DNA (Promega, Madison WI, USA) purification protocol, which was adapted to use 0·4 g of starting material (Bartley *et al*. 2013).

Detection of protozoan DNA by 18S PCR and ITS1 PCR

Parasite DNA was detected using a nested PCR, targeting the multi-copy 18S rDNA of the ribosomal RNA gene family. The first round PCR used external primers that recognized various apicomplexan parasites including *Neospora caninum*, *Toxoplasma gondii* and *Sarcocystis* spp. (Table 1). Briefly, each 20 µL reaction contained 2 µL of 10× custom PCR mix- (45 mM Tris-HCl, 11 mM $(NH_4)_2SO_4$, 4·5 mM $MgCl_2$, 0·113 mg mL^{-1} BSA, 4·4 µM EDTA and 1·0 mM each of dATP, dCTP, dGTP and dTTP) (ABgene, Epsom, Surrey, UK), 0·25 pM of each primer (Eurofins MWG Operon), 0·75 units of BioTaq (Bioline, London, UK), 13·85 µL of water and 2 µL of sample DNA (Burrells *et al*. 2016). The PCR conditions for the first round were 95 °C for 5 min followed by 35 cycles at 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min, with the final extension period at 72 °C for 5 min. The primary PCR amplicons were diluted with 100 µL DNase/RNase free water and 2 µL of the diluted primary amplification product was added as template DNA for the second round amplification. Second round primers were designed to amplify only *Sarcocystis* spp. (Table 1). The specificity of the primers were tested using *S. neurona*, *S. lutrae*, *S. gigantea*, *Sarcocystis tenella*, *Sarcocystis rileyi*, *Sarcocystis fayeri*, *N. caninum* and *T. gondii* DNA samples (data not shown). The reaction conditions for the second round PCR were identical to the first round, with the exception that internal forward and reverse primers were used. Each batch of samples analysed, contained a positive control: *S. lutrae* (obtained from this study) and negative controls: *N. caninum*, *T. gondii* and water and were tested in duplicates. With each batch of badger samples extracted, a negative (water) 'extraction control' was tested (Bartley *et al*. 2013). Badgers that showed strong positive bands for both neck and tongue samples in the 18S rDNA PCR, were tested further using the ITS1 PCR. Here, Sarcocystidae were detected using the primers 'SU1F' and '5.8SR2' that amplify the ITS1 region (~1000 bp) and targets the adjacent 18S and 5.8S rDNA genes, respectively (Gjerde, 2014) (Table 1). The reaction conditions for the ITS1 PCR were identical to those of the 18S rDNA PCR. The ITS1 region was selected to differentiate members of the group Sarcocystidae as it is highly polymorphic compared with the 18S rDNA. PCR products (6 µL) were analysed by 2% agarose gel electrophoresis, stained with gel red (1 : 10 000) (Biotonium, Hayward, USA) and visualised using ultraviolet light. Each batch of

Table 1. Sequences and specificity of primers used for the detection of *Sarcocystis* spp DNA in badger samples

Region	Primer	Froward	Primer	Species amplified	Reference
18S	External Forward	NTS-18S-F1	5'-GCC ATG CAT GTC TAA GTA TAA G-3'	*N. caninum*, *T. gondii* and *Sarcocystis* spp.	This study
	Reverse	NTS-18S-R1	5'-CCT ATC ATT CCA ATC ACT AGA AAT-3'		
	Internal Forward	NTS-18S-F2	5'-GGA TAA CCG TGG TAA TTC TAT G-3'	*S. lutrae*, *S. neurona* and *S. lacertae*	
	Reverse	S-18S-G9	5'-CAT CGC CGA CCA AAA AGG-3'		
ITS1	External Forward	SU1F	5'-GAT TGA GTG TTC CGG TGA ATT ATT-3'	*Sarcocystidae*	Gjerde (2014)
	Reverse	5.8SR2	5'-AAG GTG CCA TTT GCG TTC AGA A-3'		
	Internal Forward	S-ITS1-F	5'-TTT CTG TAG CGT TGA GAG GAG T-3'	*Sarcocystidae*	This study
	Reverse	S-ITS1-R	5'-CGC CTC GCT CAA CAT CAT CAT AAA-3'		

samples analysed by ITS1 PCR contained a positive control: *S. lutrae* and negative control water.

Cloning, DNA sequencing and sequence assembly

The PCR products from 12 animals (both positives for tongue ($n = 12$) and neck muscle ($n = 12$)) using the 18S external primers and six positive PCR products from four animals (positives tongue ($n = 4$) and neck ($n = 2$)) using the external ITS1 PCR, were purified using the commercially available Wizard® SV Gel and PCR Clean-up System (Promega, Madison WI, USA). The PCR products were eluted in 50 μL of DNase/RNase free water and the nucleic acid concentration was determined by spectrophotometer (Nanodrop, ND1000). For each sample, 100 ng of DNA was sent for sequencing (Eurofins MWG Operon). The 18S amplicons were sequenced with the 18S primers and the ITS1 amplicons were sequenced with the ITS1 primers (Table 1).

Three first round PCR amplicons from the 18S rDNA PCR (tongue $n = 1$, neck $n = 2$) and a further 3 PCR amplicons from the ITS1 PCR (tongue $n = 2$, neck $n = 1$) were cloned using the pGEM®-T Easy Vector System (Promega, Madison WI, USA) as previously described (Bartley *et al*. 2016) with the following alterations. Two microliter (64 ng) of the purified product were ligated into the pGEM®-T Easy Vector (1 μL at 50 ng μL^{-1}) (Promega, Madison WI, USA) according to the manufacturer's instructions. Following ligation, 1 μL (8 ng) of ligated vector/insert was used to transform 40 μL of high-efficiency competent JM109 cells ($\geq 1 \times 10^8$ cfu μg^{-1} DNA) (Promega, Madison, WI, USA) using manufacturer's instructions. A successful transformation was confirmed using LB agar plates containing 100 μg mL^{-1} ampicillin, spread with 100 μL of IPTG (Isopropyl β-D-1-thiogalactopyranoside) (100 mM) and 20 μL of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (50 mg mL^{-1}). White colonies were screened by PCR using the 18S external primers and the SU1F and 5.8SR2 primers to confirm the presence of the *Sarcocystis* 18S rDNA and ITS1 region insert. Three clones from each of the three badger samples from each of the 18S ($n = 9$ clones) and ITS1 ($n = 9$ clones) PCR were sequenced (Eurofins, MWG Operon) using T7 and SP6 primers. Additional internal S-ITS1-F and S-ITS1-R primers (Table 1) were used for the ITS1 clones to ensure a double stranded consensus sequence of over 1000 bp was generated. Overall consensus sequences were generated for the 18S and ITS1 amplicons from each badger.

A Basic Local Alignment Search Tool (BLAST) search was completed to determine percentage identity of the generated sequences against previously published sequences. Multiple sequence alignments were performed using the BioEdit sequence

alignment editor 7.1.3.0. to show the difference between the closely related *Sarcocystis* spp. Phylogenetic analyses were performed on both the 18S rDNA and ITS1 consensus sequences using MEGA6 software (Tamura *et al*. 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura–Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained by applying the Neighbour–Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and all positions containing gaps and missing data were eliminated. The phylogeny was tested with the bootstrap method, using 1000 bootstrap replications.

Statistical analysis

The proportion of positive samples (prevalence), with confidence intervals (95% CI) was calculated for the presence of *Sarcocystis* DNA in the tongue and neck muscle samples from badgers. The numbers of badgers where either tongue, or neck muscle sample were positive and those animals where both samples were positive were also calculated. All of the calculations were carried out using the Minitab 17 software (v17.1.0.0).

RESULTS

Screening of samples for the presence of protozoan DNA using the 18S rDNA PCR

Five badgers were initially screened and DNA samples from leg, neck muscle, tongue, sub-mandibular lymph node, liver, lung, brain, heart and spleen, were tested using the 18S external primers in a single round PCR. Positive PCR amplicons were observed for 1/5 leg muscle, brain and lung sample, 2/5 neck muscle samples and 3/5 tongue and spinal cord samples. Sequencing PCR amplicons from one neck muscle and two tongue samples showed identity to *S. lutrae* (accession KM657770). The PCR products from tongue and neck were the only samples to produce identifiable sequences, and thus these organs were selected for further testing. Spinal cord was not selected for further analysis, due to the limited numbers of samples available ($n = 12$).

Verification of PCR specificity and sequencing

The *Sarcocystis* specific 18S rDNA nested PCR was used to screen all muscle samples available, tongue ($n = 32$) and neck muscle samples ($n = 54$) from 54 badgers. The results showed that 36/54 (67%) (95% CI: 52·5–78·9%) neck samples and 24/32 (75%) (95% CI: 56·5–88·5%) tongue samples tested positive for *Sarcocystis* DNA. Twenty badger samples showed positive PCR results for *Sarcocystis* DNA in both neck and tongue samples using the 18S rDNA PCR (20/32) (95% CI: 43·6–78·9%). Forty badgers tested positive with the *Sarcocystis* specific 18S rDNA PCR with at least one tissue (40/54, 74%) (95% CI: 60·3–85·0%). No amplified products were observed for the negative controls: water, *T. gondii* and *N. caninum*. Badgers ($n = 20$) that showed positive results for both tongue and neck in the 18S rDNA PCR were tested using the ITS1 PCR (Gjerde, 2014). The ITS1 PCR revealed positive results for 12/20 (60%) (95% CI: 36·0–80·8%) neck and 10/20 (50%) (95% CI: 27·1–72·8%) tongue samples for *Sarcocystidae* DNA. No PCR amplicons were generated for 4/20 badgers tested using the ITS1 primers.

Consensus sequences were generated for the 18S rDNA from 9 clones: tongue ($n = 3$) and neck ($n = 6$) and for the ITS1 region from 9 clones: neck ($n = 3$); and tongue ($n = 6$). These clones were used to create consensus sequences for the 18S rDNA and ITS1 amplicons for each animal. The 3 consensus sequences, each for the 18S rDNA and the ITS1 amplicons, were identical to each other and were used to create a general consensus sequence for both the 18S rDNA and ITS1 regions. The general consensus sequences for the 18S rDNA (468 bp) and the ITS1 region (1074 bp) were submitted to Genbank (KX229728 and KX431307, respectively). When the 18S rDNA (KX229728) and ITS1 (KX431307) sequences generated during this study, were compared on NCBI BLAST against published DNA sequences, it was found that the 18S rDNA fragments showed 100% identity to isolates of *S. lutrae* (18S rDNA: KM657770). The ITS1 sequence showed 99·2–100% identity to the 22 ITS1 sequences of *S. lutrae* found in the Eurasian otter (*Lutra lutra*) (Gjerde and Josefsen, 2015).

Phylogenetic relationship and multiple sequence alignments of S. lutrae *and related species*

Phylogenetic analysis revealed that the *S. lutrae* rDNA found in badgers appears in the same clade as the *S. lutrae* found in otters, as well as the closely related species *S. nileyi* and *Sarcocystis turdusi* (Fig. 1A). The multiple sequence comparison demonstrated that the 18S rDNA fragment found in badgers (KX229728) is identical to *S. lutrae* found in otters (KM657775) (Fig. 2A). The sequence alignment of the 18S rDNA (Fig. 2A) shows polymorphic and conserved regions for the closely related *Sarcocystis* species. The alignment shows that our sequence and *S. lutrae* are identical to each other but are distinct from the other closely related species sequences by one additional "T" base in comparison with *Sarcocystis corvusi* and *S. arctica* and *Sarcocystis turdusi*, and multiple base

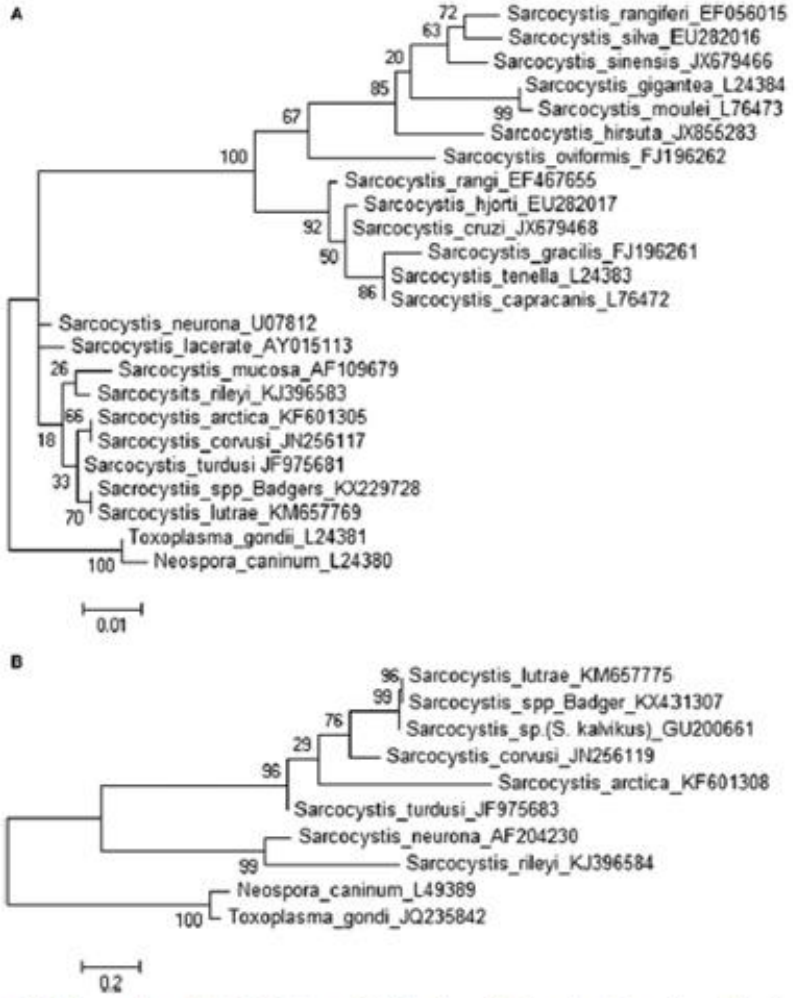

Fig. 1. Molecular Phylogenetic analysis by Maximum Likelihood method for selected members of the *Sarcocystidae*. (A) 18S rDNA with the highest log likelihood (−1280·9024) and (B) ITS1 spacer region with the highest log likelihood (−2396·9991). The percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

pair differences from *S. rileyi*, *Sarcocystis lacerate*, *Sarcocystis mucosa* and *Sarcocystis neurona* (Fig. 2A). The ITS1 region was also used for sequence alignments and phylogenetic analysis, since the 18S rDNA gave poor discrimination of closely related species. The ITS1 phylogenetic analysis showed a clearer differentiation from the closely related *Sarcocystis* spp. (*S. corvusi*, *S. arctica*, *S. neurona*, *S. turdusi* and *S. rileyi*), however *Sarcocystis kalvikus* was found in the same clade as *S. lutrae* from badgers and *S. lutrae* from otters (Fig. 1B). Yet, when using the ITS1 multiple sequence analysis, it can be seen that *S. kalvikus* can be distinguished from the *S. lutrae* found in badgers and *S. lutrae* found in otters. The ITS1 region is more polymorphic compared with the 18S rDNA and the ITS1 sequence comparison showed a clear differentiation of *S. lutrae*, *S. kalvikus*, *S. turdusi*, *S. corvusi* and *S. arctica* (Fig. 2B). From the multiple sequence analysis of both the 18S rDNA and ITS1 regions, it can be clearly seen that the *Sarcocystis* spp. rDNA fragments found in the sample of badgers in this study are identical to the *S. lutrae* found in otters.

DISCUSSION

In this paper we report the detection of 18S rDNA and ITS1 region in tongue and neck muscles of European badgers (*M. meles*) collected from around

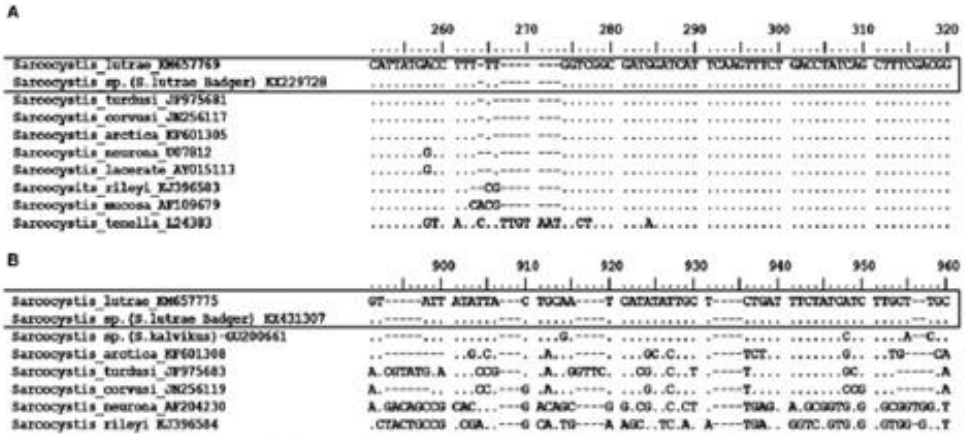

Fig. 2. Multiple sequence alignment of the polymorphic sections of the 18S and the ITS1 region amplified in this study: (A) 18S rRNA gene region, (B) ITS1 region. Boxing shows identical sequences of *S. lutrae* (18S KM657769 and ITS1 KM657775) and the Sarcocystis sequence detected in the badger samples (18S KX229728 and ITS1 KX431307). Dots represent identical base pairs and dashed lines represent gaps in the alignments. Numbers given above each alignment correspond to the nucleotide position in the sequence (A) KM657769 and (B) KM657775, respectively.

the Lothians and Borders regions of Scotland, that show 100% sequence identity to DNA from *S. lutrae* (KM657770 and KM657775). *Sarcocystis lutrae* has previously been identified in tongue, skeletal muscle and diaphragm in the Eurasian otter (*Lutra lutra*) (Gjerde and Josefsen, 2015). Moreover, Gjerde and Schulze (2014), found that the cox1 sequence of *S. lutrae* from otters were identical with one of the cox1 sequence from an arctic fox harbouring *S. arctica*. One of those cox1 sequences was initially assigned to *S. arctica* even though it differed slightly from the other cox1 sequences obtained and was later re-assigned to *S. lutrae* and thus the presence of *S. lutrae* in Arctic foxes can be disputed (Gjerde and Josefsen, 2015). This study used the 18S rDNA and ITS1 region, to verify and identify the 18S rDNA fragments found in badger samples. Phylogenetic and multiple sequence alignments have shown that the ITS1 region is more polymorphic, compared with the 18S rDNA gene. Using multiple loci, such as the 18S rDNA gene and the ITS1 region will help species identification and is more reliable than using one locus alone. Few polymorphic regions in the 18S rDNA gene have previously been shown in especially closely related *Sarcocystis* species (i.e. *S. lutrae*, *S. turdusi*, *S. arctica*, *Sarcocystis wobeseri*) and little sequence data has been generated. It has been shown that the ITS1 region gives a clearer differentiation for these species (Gjerde and Schulze, 2014; Gjerde and Josefsen, 2015). Since the ITS1 region is not a gene, higher mutation densities are tolerated, making this region highly variable among species and thus a useful marker for species identification for some, however, not all *Sarcocystis* spp.

From this study it can be confirmed that the 18S rDNA and ITS1 region identified in badgers showed 100% sequence identity to *Sarcocystis lutrae* (KM657770 and KM657775), indicating that the *Sarcocystis* species detected is likely to be *S. lutrae*. Active infections may have been detected if LM of fresh muscle tissue, such as tongue muscle, were analysed. *S. hofmanni*, *S. melis*, *S. cf. sebeki*, *S. cf. gracilis* and an unnamed *Sarcocystis* species have previously been recorded in European badgers (*M. meles*) and Japanese badgers (*Meles anakuma*) using TEM and, LM of fresh muscle tissue (Kubo *et al.* 2009; Odening *et al.* 1994*a*, *b*). Since those studies were conducted before molecular techniques were used in such research, no 18S or ITS1 sequences were generated for these species. Testing both neck muscle and tongue for the detection of *Sarcocystis* DNA proved advisable, as both these tissues showed a high presence of *Sarcocystis* DNA and if only one tissue was tested the overall prevalence would have been lower. The density of sarcocysts may vary in different types of muscle tissues, such as the diaphragm, oesophagus, tongue and heart. These tissues are commonly used to demonstrate the presence of sarcocysts in hosts (Dubey *et al.* 2015).

Identification of *Sarcocystis* species based on morphology employs looking at structural characteristics, such as sarcocyst wall and morphology; however, more than one *Sarcocystis* spp. may have the same sarcocysts morphology and the same species can occur in different hosts (Dubey *et al.* 1989, 2015). More recently molecular methods of *Sarcocystis* spp. have been particular useful to distinguish between morphologically indistinguishable

species in closely related intermediate hosts, such as water buffaloes and cattle, and different cervids. Ideally, individual sarcocysts should be excised from fresh muscle tissue, examined in wet mounts by LM, used to extract DNA for molecular characterization and fixed to study them using TEM. Using both identification methods would allow phenotypic and genotypic data to be combined and be linked to the species description. Using morphological characteristics alone for the identification may prove difficult as size and shape is subject to change depending on the age of the Sarcocysts but this observation can be strengthened by sequencing DNA amplicons from different regions of the parasite genome (Dubey *et al*. 2015).

The data presented in this study shows that the DNA detected in European badgers showed sequence identity at two different loci to *S. lutrae* found in otters. This shows that badgers from the Lothians and Borders regions of Scotland are frequently infected with *S. lutrae*. Badgers are omnivores, and it is likely that they become infected through the ingestion of sporocysts shed by definitive host (predator/scavenger) (Dubey and Lindsay, 2006). Birds, such as the white-tailed (sea) eagle (*Haliaeetus albicilla*) suggested by (Gjerde and Josefsen, 2015) or birds of the family *Corvidae*, as well as other badgers and foxes (*Vulpes vulpes*) may act as a definitive host of *S. lutrae*. Further research, involving microscopic analysis, as well as multiple locus sequence typing, is needed to confirm whether *S. lutrae* is widely distributed across Great Britain and whether *S. lutrae* is only found in badgers, (Eurasian) otters and potentially arctic foxes.

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